Eesti Maaülikool
Estonian University of Life Sciences

EXPOSURE EFFECTS OF SYNTHETIC AND BIOLOGICAL PESTICIDES ON HONEY BEES AND BUMBLE BEES

SÜNTEETILISTE JA BIOLOOGILISTE PESTITSIIDIDE MÕJUD MEEMESILASTELE JA KIMALASTELE

RISTO RAIMETS

A Thesis
for applying for the degree of Doctor of Philosophy
in Agriculture

Väitekiri
Filosoofiadoktori kraadi taotlemiseks
põllumajanduse erialal

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LIST OF ORIGINAL PUBLICATIONS

The present thesis is a review of the following research papers, which are referred by Roman numerals in the text.

I **Raimets, R**., Mänd, M., Bontšutšnaja, A., Bartkevics, V., Pugajeva, I., Kaart, T., Puusepp, L., Pihlik, P., Keres, I., Viinalass, H., Karise, R. 2019. Pesticide residues in beehive matrices are dependent on collection time and matrix type but independent of proportion of foraged oilseed rape and agricultural land in foraging territory. Chemosphere (accepted).

II Karise, R., **Raimets, R**., Bartkevics, V., Pugajeva, I., Pihlik, P., Keres, I., Williams, I.H., Viinalass, H., Mänd, M. 2017. Are pesticide residues in honey related to oilseed rape treatments? Chemosphere, 188, 389-396.

III **Raimets, R**., Naudi, S., Bartkevics, V,. Pugajeva, I., Mänd, M., Karise, R. 2019. Field relevant concentrations of fungicide and an insecticide are affecting honey bee (*Apis mellifera*) queens. Submited to Apidologie.

IV **Raimets, R**., Karise, R., Mänd, M., Kaart, T., Ponting, S., Song, J., Cresswell, J.E. 2018. Synergistic interactions between a variety of insecticides and an EBI fungicide in dietary exposures of bumble bees (*Bombus terrestris* L.). Pest Management Science, 74, 541-546.

V Karise, R., **Raimets, R**., Dreyersdorff, G., Mänd, M. 2018. Using respiratory physiology techniques in assessments of pesticide effects on bees. Hazards of pesticides to bees 13th International Symposium of the ICP-PR Bee Protection Group 18-20. October 2017, Valencia (Spain) - Proceedings - (61-66).

Table 1. Authors contributions to the papers

Paper	Idea and study design	Laboratory work	Data analysis	Manuscript preparation
I	**RR**, RK, MM	**RR**, RK, AB, VB, IP, LP, PP, IK, HV	**RR,** TK, RK, MM	**RR**, RK, MM, VB, AB
II	RK, **RR**, MM	**RR**, RK, VB, IP, IK, PP, HV	**RR**, VB	RK, **RR**, MM, VB, IHW
III	**RR**, RK, MM	**RR**, SN, VB	**RR**, RK, SN, MM	**RR**, RK, MM
IV	**RR**, JEC	**RR**, SP, JS, JEC	TK, RK, **RR**	**RR**, RK, MM, JEC
V	RK, MM, **RR**	**RR**, GD, RK	RK, MM, **RR**	RK, **RR**, GD, MM

RR - Risto Raimets; RK - Reet Karise; MM - Marika Mänd; SN - Sigmar Naudi; AB - Anna Bontšnutšnaja; GD - Gerit Dreyersdorff; LP - Liisa Puusepp; PP - Priit Pihlik; IK - Indrek Keres; HV - Haldja Viinalass; TK - Tanel Kaart; IHW - Ingrid H. Williams; VB - Vadims Bartkevics; IP - Iveta Pugajeva; SP - Sally Ponting; JS - Jimao Song; JEC - James E. Cresswell

ABBREVIATIONS

ABPV – acute bee paralysis virus
ANOVA – one-way analysis of variance
BPA – binomial proportion test for additivity
CCD – colony collapse disorder
CBPV – chronic bee paralysis virus
DGE – discontinuous gas exchange
DWV – deformed wing virus
EBI – ergosterol biosynthesis inhibitor
GM – genetically modified organism
IAPV – Israeli acute paralysis virus
nAChR – nicotinic acetylcholine receptor
MCPA – 2-methyl-4-chlorophenoxyacetic acid
MR – metabolic rate
PPP – plant protection product
RI – resistance index
RJ – royal jelly
SBV – sacbrood virus
SHB – small hive beetle
U.S. – United States of America
VCO_2 – volume of carbon dioxide
VH_2O – volume of water
WLR – water loss rate

1. INTRODUCTION

Insect pollination service is a very profitable tool to promote agricultural and wild plant reproduction. It is noteworthy that from 87 globally used main food crops, pollinators are essential for 13 and another 30 crops are highly pollinator dependent *(*Klein et al., 2007). Honey bees are considered one of the most efficient pollinators due to their specific foraging behaviour and vast numbers of members in a single colony (Abou-Shaara, 2014). For instance, in California, almost 2 million honey bee (*Apis mellifera* L.) colonies are used in almond orchards to provide sufficient pollination for almond trees during blooming (Traynor, 2017).

It is important to calculate pollination services into relevant numbers for a better overview of its importance. In 2005, the global economic value of insect pollination was approximately 152.9 billion euros. In Europe, this number was 14.2 billion euros (Gallai et al., 2009). In the U.S. in 2009, the value of annual insect pollination was 15.12 billion dollars (Calderone, 2012). In addition, the demand for pollination services will increase in time, given that cultivated areas of pollinator dependent crops will increase with time (Aizen et al., 2008).

Despite the importance of pollinators, their numbers are in decline. In the case of honey bees, the first alarming signals of decline were detected in 2006, when surveyed beekeepers from the U.S. lost on avarage 37.6% of their colonies (vanEngelsdorp et al., 2007). Year later, colony losses remained high, ranging between 17 – 56% in different states (vanEngelsdorp et al., 2008). At the same time, different European countries also experienced high honey bee colony winter losses. For instance, apiculturists from Italy and Finland who participated in a survey reported that they have lost 29.8% and 19.6% of colonies in winter 2009 and 2010 respectively (Zee et al., 2012). To compensate for winter losses, beekeepers split their colonies in spring to recover the number of colonies they need, though in the long-term this is not sustainable due to weakening of the main colonies just before the main honey flow.

Besides honey bees, many wild pollinators are in decline. Cameron et al. (2011) showed that, in the U.S., the abundance of four bumble bee species (*Bombus occidentalis* L.*, B. pensylvanicus* De Geer*, B. affinis* Cresson and *B. terricola* Kirby) had declined significantly in the previous two decades. Wild pollinator decline is also a problem in Europe. In

Great Britain, serious declines of six bumble bee species have been reported since the 1960s (Williams and Osborne, 2009). In addition, several bumble bee species are in decline or critically endagered in different European countries (Kosior et al., 2007). Documenting the status and trends of wild bee populations is not easy due to the absence of historical reference data and serious work load needed for observations. Also, massive death events remain unknown for wild bees. Protecting wild bees can occur only through enhancing their habitats and eliminating any known and potential stress factors (Goulson, 2003). These methods simultaneously aid both wild and managed bee species.

Beekeepers and scientists have proposed several causes to explain bee declines: GM crops, climate change, poor nutrition, habitat loss, cooled brood, low genetic diversity within bee populations, parasites and predators, various diseases and the intensive use of pesticides (Brodschneider and Crailsheim, 2010; Darrouzet et al., 2015; Evans et al., 2000; Forsgren, 2010; Goulson et al., 2015; Krongdang et al., 2018; Oldroyd, 2007; Rosenkranz et al., 2010; Sinpoo et al., 2018). Agricultural intensification goes hand-in-hand with increased pesticide inputs on fields, and thus bees visiting these crops and weeds are more likely to be exposed to different chemicals. Indeed, honey bee products often contain different pesticide residues. Mullin et al. (2010) has found altogether 98 different pesticide residues and metabolites from pollen samples collected from North American apiaries. Different pesticide residues have also been found in other bee products like honey, wax, beebread and the bees themselves (Al Naggar et al., 2015; Chauzat and Faucon, 2007; Ravoet et al., 2015; Škerl et al., 2009). In France, 19 different pesticide residues were found from pollen loads of honey bees, and among all these chemicals, the insecticides tau-fluvalinate and coumaphos were the most concentrated substances (Chauzat et al., 2006). In addition, both previously mentioned insecticides are used in apiculture as acaricides in treating varroatosis, and therefore the concentrations of these chemicals are likely to further increase (Haarmann et al., 2002).

Pesticides can affect every member of a bee colony. While comparing different acaricides' toxicity to honey bees, the LD_{50} of tau-fluvalinate has shown to be most toxic to honey bees 48 h post-treatment (Gashout et al., 2018). Even honey bee queens, who are pretty well protected from xenobiotic compounds, can be harmed by pesticides. The acaricides tau-fluvalinate and coumaphos, which are most commonly used in

Varroa treatments, have shown to decrease queen weight and longevity (Haarmann et al., 2002). Sublethal doses of pesticides evoke observable changes in bee behaviour but may cause minor changes also in physiology, which still can affect the longevity of individuals or colonies. Muljar et al. (2012) showed the effect of sublethal concentrations of the pyrethroid alpha-cypermethrin on bumble bee (*B. terrestris* L.) respiratory patterns and demonstrated the ability to determine concentrations with reversible or irreversible effects.

Throughout their life, bees encounter a mixture of pesticides. Some pesticide mixtures are more hazardous to bees than these substances alone. It is well documented that insecticide-fungicide (so called azole-type fungicides) combinations increase bee mortality significantly (Johnson et al., 2013; Sgolastra et al., 2017). It has been also shown that different pesticide cocktails are very toxic to honey bee larvae (Zhu et al., 2014).

While considering synthetic pesticides and their mixtures, and their negative effects on bees, potential alternatives have been proposed. To diminish the cost of using biological preparations, various technologies are being developed. Entomovectoring technology uses bees to deliver powdered preparations onto flowering crops (Karise et al., 2016a). However, biological preparations also require risk assessment for vectoring bees and other non-target organisms.

Considering the importance of bees in our ecosystems, their vulnerability and persistent decline, the present work investigated pesticide residues in different bee matrices and the impact of these small concentrations on different parameters among two bee species. In addition, the health risks of microbiological preparations on honey bees and bumble bees were investigated. In order to adequately assess about the health risks to bees, it is vital to understand the regional peculiarities of the actual pesticide contamination level in the bees' environment. The outcome of this work should give a better understanding of the precise (botanical) origin of different pesticide residues and how these field realistic concentrations of pesticides and their mixtures are affecting bees, as well as whether there are any non-hazardous alternatives.

2. REVIEW OF THE LITERATURE

2.1. Bee decline

Different continents have experienced severe bee decline. Since 1961, in Europe and the U.S., the number of managed honey bee colonies has decreased by 26.5% and 49.5%, respectively (FAO, 2009). Not only managed honey bees are in decline; there are concerning facts regarding wild pollinator decline. In Europe, there has been severe decline in certain bumble bee species since the 1950s, and even four species extinctions have been recorded during this period (Kosior et al., 2007). Bumble bee species richness and abundance has also decreased in North America, where *B. affinis*, *B. terricola* and *B. pensylvanicus* numbers show clear declines in different states (Grixti et al., 2009).

Bee decline is an ongoing process and a major issue nowadays. In 21st century, the first severe alarming signals came from U.S. beekeepers in winter 2006/2007 who reported abnormally high colony losses. The beekeepers who participated in the survey lost 31.8% of their colonies in total (vanEngelsdorp et al., 2007). The abnormal situation, that there were no dead or alive bees left in the hive, or just a honey bee queen with few attendants present, was termed colony collapse disorder (CCD) (vanEngelsdorp et al., 2009). The next season, the surveyed apiculturists from the U.S. lost in total 35.8% of their colonies (vanEngelsdorp et al., 2008). The high mortality continued: in 2008/2009, the winter losses in the U.S. were 29% on average, which still exceeded the acceptable colony loss level of 17.6% (the maximum rate that surveyed beekepers considered acceptable) (vanEngelsdorp et al., 2010). Bee decline has also spread to South-America. Requier et al. (2018a) showed that surveyed beekepers lost 15.5% of their colonies in Argentina in 2015/2016.

European beekeepers are also experiencing high colony losses. Beekeepers from the Netherlands and Sweden respectively lost 21.7% and 14.6% of their colonies in winter 2008/2009, while a year later (winter 2009/2010) these numbers increased to 27.8% and 28.5%, respectively (Zee et al., 2012). In winter 2016/2017, Austrian and Belgian beekeepers experienced 23.4% colony losses (Brodschneider et al., 2018). Estonian beekeepers experienced high colony losses in winter 2012/2013: small beekeeping operations with 1-50 colonies lost 24.4% of honey bee

colonies on average; professional beekeepers with 151 or more colonies lost 27.2% of colonies (Zee et al., 2014).

In recent years, the percentage of winter losses in Estonia remained stable but still over the acceptable level (up to 10%). According to COLOSS survey, the colony winter loss rates in Estonia in 2014/2015 were 19.3%, and two years later (2016/2017) this number decreased to 13.4% (Estonian Apicultural Program, 2017). Gray et al. (2019) showed that honey bee colony losses in winter 2017/2018, in Estonia, were 16.4% on average.

2.2. Potential drivers for bee decline

Bee decline can potentially be driven by various stress factors summarized in Figure 1. Habitat loss and poor nutrition has been proposed as main potential causes of wild and managed bee decline (Brodschneider and Crailsheim, 2010; Goulson et al., 2015). In addition, the spread of different parasites and predators are likely also reasons. There are also other stress factors like direct pesticide exposure and residues in bee food (Kasiotis et al., 2014; Fulton et al., 2019), genetically modified (GM) crops and low genetic diversity within bee populations (Oldroyd, 2007), which may contribute to bee mortality.

Due to agricultural intensification and peculiarities of habitat and climate, bees sometimes experience nutritional stress, which may amplify the impact of other stress factors (Huang, 2012; Scofield and Mattila, 2015). It has been shown that habitat loss and poor nutrition may lead to metabolic stress in honey bees, and thus due to lower energetic base their probability of returning from foraging decreases significantly (Naug, 2009). Habitat loss has shown to negatively affect wild bee communities as well, including even generalist pollinators populations, despite they should be better adapted to changing conditions (Bommarco et al., 2010).

The parasitic mite *Varroa destructor* Anderson and Trueman has been considered one of the most substantial single drivers for honey bee declines globally (Rosenkranz et al., 2010). *Varroa* mites have spread almost all over the world, except Australia and some African countries (Iwasaki et al., 2015). Adult mites feed on haemolymph from honey bees, and simultaneously vector different viruses like deformed wing virus (DWV) and chronic bee paralysis virus (CBPV) (Le Conte et al., 2010; Rosenk-

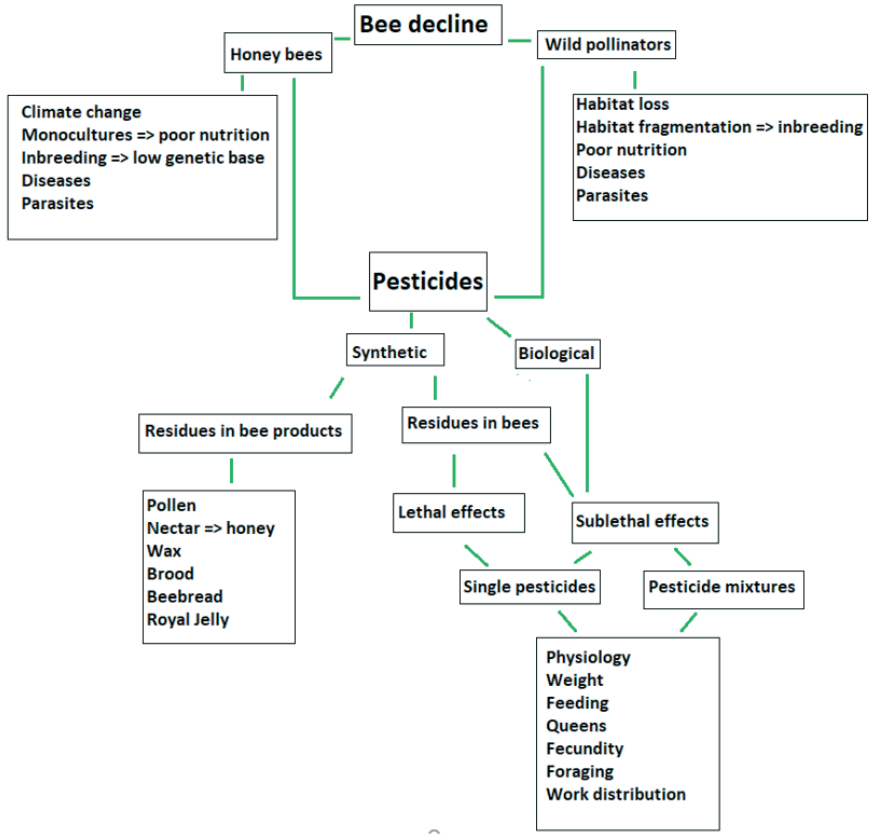

Figure 1. Potential drivers for managed and wild bee decline.

ranz et al., 2010), acute bee paralysis virus (ABPV), sacbrood virus (SBV) and Israeli acute paralysis virus (IAPV) (Boecking and Genersch, 2008).

Some other arthropod pests can be detrimental to bee colonies. For instance, small hive beetle (*Aethina tumida* Murray) (SHB) larvae feed on pollen, honey and brood in wax combs, thus causing whole comb collapse (Evans et al., 2000). Highly infested honey bee colonies leave their hives, thus favouring SHB distribution (Hood, 2004). It has been observed that SHB can successfully use bumble bee (*B. impatiens* Cresson) colonies as a host in which SHB oviposits (Hoffmann et al., 2008), thus wild bees are also potentially under threat. Another insect, the yellow-legged Asian hornet *Vespa velutina* Lepeletier is an additional threat to European honey bees (Requier et al., 2018b). *V. velutina*, which feeds on forager bees, has expanded its territory across several European countries (Darrouzet et al., 2015; Keeling et al., 2017; Requier et al., 2018b; Robinet et al., 2017).

These hornets actively hunt honey bees during summer and autumn, when hornets rear their brood (Monceau et al., 2013).

Like most living organisms, bees are exposed to various diseases. Nosematosis is a very common honey bee disease which can be caused by two different species of microsporidia: *Nosema apis* Zander and *N. ceranae* Fries (Sinpoo et al., 2018). Both nosema species elicit increased mortality in *A. mellifera* and *A. cerana* (Sinpoo et al., 2018). The clinical symptoms of *N. apis* presence in a bee colony include diarrhea and a great number of dead bees in the hive (Bourgeois et al., 2010). In the case of *N. ceranae*, no visible symptoms exist except for the steadily increasing mortality within the colony (Bourgeois et al., 2010). Also, wild bees have their own nosematosis. There is a study showing that *B. terrestris* larvae and adults were both susceptible to *N. bombi* Fantham and Porter. Interestingly, the same study shows that *N. bombi* was significantly less lethal to *B. terrestris* than two other bumble bee hosts (*B. lapidarius* L. and *B. hypnorum* L.) (Schmid-Hempel and Loosli, 1998).

The bacteria American foulbrood (AFB) (*Paenibacillus larvae*) and European foulbrood (EFB) (*Melissoccus plutonius*) are both lethal to honey bee larvae (Forsgren, 2010; Krongdang et al., 2018). They are both widespread in Europe as well as the U.S. (Ellis and Munn, 2005; vanEngelsdorp and Meixner, 2010). These diseases are so severe to honey bees that the control is regulated by national laws.

Honey bees tend to suffer even more when they are exposed to additional stress factors like pesticides or low-quality food. For instance, environmental contamination by pesticides may increase bee mortality and decrease tolerance to diseases (Aufauvre et al., 2012; Brandt et al., 2016). Despite the myriad of studies on the pesticide residue levels in hives, effects of single chemicals on bee health or certain diseases, there is still a gap in knowledge regarding how various stress factors which bees are exposed to may interact with each other. For example, there is still no clear explanation for CCD, but rather various causes have been proposed (vanEngelsdorp et al., 2008, 2009).

2.3. Pesticides

For farmers, pesticides are essential to protect their crops against weeds, pests and diseases. The aforementioned factors can cause 26% - 80%

yield loss for farmers (Oerke, 2006), and thus it is often necessary to use plant protection products (PPP). In general, pesticides can be divided into 3 main classes: insecticides, fungicides and herbicides (Yadav and Devi, 2017). All of them can be either synthetic or non-synthetic, depending on the method of production. Non-synthetic pesticides are natural products with lower environmental risk, but have often lower efficacy than synthetic compounds (Robin and Marchand, 2019). Therefore the use of synthetic pesticides is prevailing, despite the accompanying environmental risks.

2.3.1. Multiple routes of pesticide exposure for bees

Bees can be exposed to pesticides in various ways. However, the most common source for pesticide exposure is the agriculture. In agriculture, bees can be exposed to chemicals via direct spraying, seed coating, contaminated guttation water, dried pesticide residues or already contaminated dust accompanying sowing (Kiljanek et al., 2016). Due to spray drift , the bees are likely exposed to pesticides in neighbouring areas as well (Blanco et al., 2019). Pesticide drift from initial field was confirmed in a study where bees collected from grasslands near agricultural fields contained significant amounts of residues of different pesticides (Hladik et al., 2016). Forager bees may also be exposed to pesticides while foraging on weeds flowering in fields or on the other land use types that are treated with pesticides (Larson et al., 2013). Similarly, in urban areas, people treat their lawns and flowerbeds against weeds or pests, and hence put the bees at risk.

Seed coating techniques eliminate spray drift, but is still hazardous to bees because systemic compounds can end up in nectar and pollen (Girolami et al., 2009). In addition, it has been shown that from seed coating, only 1.6 - 20% of imidacloprid entered the seed, the remaining leakeing out to the surrounding environment (Sur et al., 2003). Pesticide residues in soil can be easily picked up by surface water and transported to neighbouring areas where it is absorbed by the roots of other plants, increasing the possibility of non-target plants becoming contaminated. A study conducted in North America clearly shows pesticides persistence and drift in soil after the sowing of treated maize seed (Krupke et al., 2012). They also found pesticide residues from bee forage plants growing near the agricultural fields.

It is noteworthy that several pesticide reidues in beehives originate from apiculture itself. Beekeepers use different acaricides like tau-fluvalinate, coumpahos and amitraz to treat colonies against the *Varroa* mite (Elzen et al., 2000; Haarmann et al., 2002). These acaricides are lipophilic, resulting in direct accumulation into honey bee wax (Chauzat and Faucon, 2007; Ravoet et al., 2015), causing long-term exposure.

2.3.2. Contamination in bee colony components

The intensification of agriculture increases bee exposure to pesticides. There are several publications showing honey bee contamination. A study from Slovenia shows that pollen and beebread samples collected from honey bee hives near apple orchards were contaminated by insecticides and fungicides used there (Škerl et al., 2009). Pollen from French apiaries were contaminated by various pesticides from different classes including fungicides and insecticides (Lambert et al., 2013). A 3-year survey in Italy showed that honey bee collected pollen is highly contaminated by pesticides across the country; 62% of the 554 pollen samples collected contained at least one pesticide residue, and 38% of samples were contaminated with more than one pesticide (Tosi et al., 2018). It is notable that in North America up to 31 different pesticide residues have been found from a single pollen sample, and residues of an average of 7.1 pesticides were found in each pollen sample (Mullin et al., 2010). Pesticide residues found from different bee matrices can indicate the actual pesticide usage on fields.

Not only pollen and beebread from beehives are contaminated by pesticides. Several insecticide residues have been found from honey in several countries, see review by (Souza Tette et al., 2016). Contamination occurs not only because of spraying; residues are also present also due to seed treatment. In a German study, unprocessed nectar collected from flying honey bee foragers was contaminated by the neonicotinoid clothianidin from oilseed rape grown from treated seed (Rolke et al., 2016). Moreover, a study performed in the United Kingdom shows that neonicotinoid pesticides are still present in honey samples after their ban in European Union. Neonicotinoid residue presence in honey was correlated with surrounding oilseed rape fields, suggesting that neonicotinoid residues can persist in soil for longer periods (Woodcock et al., 2018).

Lipophilic pesticides can easily accumulate in wax. Honey bee wax samples collected from French apiaries in different regions were conta-

minated by 14 different pesticides in total. Two of the most frequent residues, tau-fluvalinate and coumaphos, are actively used by beekeepers in apiculture to treat *Varroa* (Chauzat and Faucon, 2007). Wax from North American apiaries is highly contaminated by fluvalinate and coumaphos (Mullin et al., 2010). A recently published study shows that different fungicide residues can also be found from honey bee wax. Carbendazim, tebuconazole and thiabendazole residues were found from wax in different Western Australian apiaries (Manning, 2018). Belgian data suggests that pesticide residues can persist in wax from beehives for long time periods, since many detected contaminants became forbidden years ago (Ravoet et al., 2015).

The contamination of bees themselves may come from direct contact with pesticides on fields or from consuming contaminated food. Residues of the neonicotinoid clothianidin have been found from dead bees collected near hive entrances (Krupke et al., 2012). In Estonia, most cases of massive bee deaths come from dimethoate, one of the cheapest chemical insecticides available (Estonian Agricultural Board 2019). Dead and live honey bee samples were collected for pesticide analyses across Poland, results indicating that 20% of live bees contained traces of at least one pesticide, and 24% containing residues of multiple pesticides. At the same time, 85% of dead bees that were officially considered poisoned contained multiple pesticides, and only 1% of dead bees sampled were free of pesticides (Kiljanek et al., 2017). In Greece, the honey bee forager samples were collected from different locations for pesticide residue analysis in three consecutive years. The results revealed that in all monitored years the collected bee samples contained pesticide residues from different classes (Kasiotis et al., 2014).

Not only honey bees are exposed to different pesticide residues. Wild pollinators like bumble bees forage on various plant taxa that may be contaminated by pesticides. Also it has been shown that bumble bees visit various plant taxa to fill their proper protein:lipid ratio needed (Kitaoka and Nieh, 2008; Vaudo et al., 2016), which suggests that they forage on natural plants and on mass flowering crops, and thus there is always a possibility that they may forage on both contaminated and non-contaminated plants. Due to an often small foraging radius, many solitary bees forage on plants grown nearby (Gathmann and Tscharntke, 2002), which in turn means that they may not have a choice between contaminated or non-contaminated plants. A study shows that native

bees collected from wheat fields and grasslands were contaminated by various pesticides in two consecutive years (Hladik et al., 2016), confirming the small foraging radius of solitary bees, as well as pesticide drift from the wheat fields.

2.3.3. Impact of pesticides on bees

Pesticides can affect bees in various ways. First, pesticides can have direct lethal effects on bees (Calatayud-Vernich et al., 2016; Laurino et al., 2013; Muljar et al., 2012), and pesticide labels include information about the LD_{50} values for many non-target organisms. Second, pesticides can cause sublethal effects in bees, which often cannot be observed by the human eye. The herbicide glyphosate has been shown to negatively affect honey bee navigation, which was probably caused by glyphosate inhibition of bee cognitive capacities like learning and memory (Balbuena et al., 2015). The biopesticide kaolin increased bumble bee (*B. terrestris*) water loss rate significantly (Karise et al., 2016b). Pyrethroid insecticides have been shown to have deleterious effects on insect muscle activity and metabolic rate (Mänd and Karise, 2015). Pesticides have also been shown to negatively affect bumble bee feeding rate, which resulted in decreased amount of consumed food (Laycock et al., 2014, 2012). The neonicotinoid imidacloprid has been shown to negatively affect bumble bee (*B. terrestris*) colony growth (Whitehorn et al., 2012).

2.3.3.1. Effects of single compounds

Despite the fact that pesticides from different classes are used on the fields, most of the studies are focusing on insecticides impact on bees due to their broad range of effects. Sublethal concentrations of the pyrethroid alpha-cypermethrin elicited a significant increase in bumble bee (*B. terrestris*) mortality at a higher concentration and led changes in respiratory patterns at a lower concentration (Muljar et al., 2012). This chemical is allowed to be used on flowering crops, and it is important to emphasise that not always the marked repellent effect of insecticides do not work and thus bees are more likely exposed to chemicals. A study shows that spring oilseed rape freshly treated with alpha-cypermethrin had a significantly higher number of bees per flowering unit (Karise et al., 2007). Bumble bee pollen foraging was disrupted when they were fed with environmentally relevant concentrations of the neonicotinoid imidacloprid (Feltham et al., 2014). Field realistic concentrations of imi-

dacloprid have also been shown to affect bumble bee (*B. terrestris*) fecundity (Laycock et al., 2012). The neonicotinoid insecticides acetamiprid, clothianidin, thiacloprid and thiametoxam have been shown to increase honey bee mortality, using the highest dose marked on the label (Daniela et al., 2011). Besides neonicotinoids, other studies show fipronil toxicity to honey bees, see review by (Pisa et al., 2014). In addition to mortality, the negative impact of neonicotinoids on honey bee immunocompetence has been observed. Field realistic concentrations of thiacloprid and imidacloprid decreased hemocyte density and encapsulation response in honey bees (Brandt et al., 2016). Two popular acaricides, fluvalinate and coumaphos, used in *Varroa* treatment have been shown to decrease honey bee queen weight significantly (Haarmann et al., 2002). The negative effect of coumaphos on queens has been confirmed in study conducted in the U.S. (Pettis et al., 2004).

Fungicides alone are quite non-toxic to bees, and thus their potential risk to bees is considered rather low. The impact of the fungicide Captan (formulation Captan 50 WP EPA reg. no. 51036-166 or Captan 80 WDG EPA reg. no. 66222-58-51036) on honey bee colony health and brood development was investigated, and it turned out that a field realistic concetration of Captan in almond orchards did not negatively affect the honey bee parameters measured (Everich et al., 2009). Ladurner et al., (2005) tested effects of five formulated fungicides (benomyl, captan, iprodione, propiconazole and neem oil) on *A. mellifera* and *Osmia lignaria* Say survival. Only the fungicide Captan decreased *O. lignaria* survival significantly. Nevertheless, Simon-Delso et al. (2018) showed that fungicide boscalid toxic effects on honey bees occurred after 10 days, which means that the methodology of fungicides toxicity tests should be reconsidered.

Herbicides also are not very toxic to bees but can detrimentally affect other bee parameters. A study shows that sublethal doses of herbicide glyphosate can slightly affect honey bee acetylcholinesterase (AchE) activity, and therefore may lead to changes in bee general activity and homeostasis (Boily et al., 2013). It can also negatively affect honey bee cognitive abilities such as navigation. Sublethal concentration of glyphosate (10 mg L^{-1}) caused navigation problems in forager honey bees. Bees spent more time returning home and performed indirect flights (Balbuena et al., 2015). In addition, because glyphosate affects microbes, it also changes the honey bee gut microbiotia (Motta et al., 2018).

Due to detrimental effects of synthetic chemicals on bees, and their persistence in environment, searching for alternatives to synthetic pesticides has received more attention. Microbiological preparations have been shown to be valuable tools in modern plant protection (Hokkanen et al., 2015; Karise et al., 2016a). Domestic honey bees and wild pollinators like bumble bees can be used as useful tools to carry powdered preparations to flowers (Hokkanen et al., 2015; Smith et al., 2012). Using bees as vectors helps to lower the cost of plant protection activities due to bee foraging behaviour. However, there are also studies focusing on the impacts of microbiological preparations or insecticidal inert materials on bees (Smagghe et al., 2013; Karise et al., 2016b). Due to the development of organic and integrated farming systems, the need for microbial preparations for open field use is increasing. Therefore, additional studies are needed to determine the sublethal effects of non-synthetic plant protection products on bees.

2.3.3.2. Effects of multiple compounds

Different pesticide mixtures may have additive, synergistic, neutral or antagonistic effects on bees. Besides chemicals belonging to same class, pesticides from different classes may have negative co-effects on bees. Different classes of pesticides may cause synergistic toxicity, which can be more deleterious to bees. Synergistic effects of fungicide-insecticide mixtures on bees were first observed last century, when the fungicide propiconazole significantly increased the toxicity of the pyrethroid lambda-cyhalothrin to honey bees (Pilling and Jepson, 1993). Bees use enzymes to detoxify xenobiotic compounds in their body, and the main enzymes involved here are called cytochrome P450 monooxygenases (Johnson et al., 2012). Johnson et al., (2013) demonstrated that a popular acaricide tau-fluvalinate and common fungicide prochloraz in combination resulted in a significantly synergistic toxicity in honey bees, probably due to the fungicide's inhibitive action on cytochrome P450 monooxygenases. However, this effect does not occur with all fungicide-insecticide interactions. The neonicotinoid thiacloprid's contact toxicity to honey bees is considered pretty low (Thompson et al., 2014), and even in combination with the ergosterol biosynthesis inhibitor (EBI) fungicide tebuconazole no synergistic detrimental effect was observed on honey bee foraging intensity and mortality (Schmuck et al., 2003). Nevertheless, environmentally relevant dosages of another neonicotinoid clothianidin, and the EBI fungicide propiconazole, in mixture, resulted in synergistic toxicity,

where mortality increased in all three bee species used (*A. mellifera, B. terrestris, O. bicornis*) (Sgolastra et al., 2017). Synergistic interactions can also occur between pesticides from the same chemical class. Two apiculturally popular acaricides, tau-fluvalinate and coumaphos, decreased honey bee resistance to xenobiotic substances. Coumaphos pre-treatment significantly decreased tau-fluvalinate detoxification capacity in honey bees (Johnson et al., 2009), confirming that pesticides even from same chemical class, when used together, can be more hazardous to bees than these pesticides used alone. In addition, the neonicotinoid imidacloprid and the pyrethroid lambda-cyhalothrin, in combination, increased bumble bee (*B. terrestris*) mortality significantly (Gill et al., 2012).

Various pesticide mixtures have been shown to be detrimental also to honey bee larvae. Four environmentally relevant dosages of fluvalinate, coumaphos, chlorpyrifos and chlorothalonil were mixed into honey bee larval diet in different combinations and fed to the larvae. The results showed that, with the fluvalinate and chlorothalonil mixture, synergistic toxicity was observed, and the same outcome was obtained in the fungicide-coumaphos mixture (Zhu et al., 2014).

2.3.4. Combination of effects of pesticides with other stress factors

As shown previously, various stress factors affect bees. Different scientific papers postulate that the decline of bee populations is caused by the combination of various stress factors (Doublet et al., 2015; Meeus et al., 2018; Nazzi and Pennacchio, 2018). Pesticide-pathogen interactions have been shown to be hazardous to bees. The combination of the pathogen *N. ceranae* and a sublethal dose of the insecticide fipronil had significantly higher negative effects on adult honey bee mortality compared to these two stress factors alone (Aufauvre et al., 2012). *N. ceranae* together with thiacloprid significantly increased adult honey bee mortality (Doublet et al., 2015). Similar results are shown in another study where a higher used dose of thiacloprid combinedwith *N. ceranae* synergised and elevated honey bee mortality substantially, while a lower thiacloprid dose did not synergise with *N.ceranae* (Retschnig et al., 2014). Newly emerged bees originating from a colony previously treated with imidacloprid were much more susceptible to the both species of pathogen *Nosema* than previously untreated bees (Pettis et al., 2012). The neonicotinoid thiacloprid increased black queen cell virus (BQCV) viral loads in honey bee larvae, and therefore the mortality increased

(Doublet et al., 2015). Still, the sublethal doses of the neonicotinoid thiacloprid and the acaricide tau-fluvalinate did not synergise with the *N. ceranae* microsporidia, but the pesticides alone increased bee mortality (Retschnig et al., 2015).

Adding poor nutrition to previously mentioned stress factors (diseases and pesticides) only enhances the possibility that bee colonies may collapse. Due to intensive agriculture and mass-flowering crops, the bee diet remains pretty often one-sided and thus lack of different proteins and vitamins may lead to weakening of bees (Goulson et al., 2015). Exposing already weakened bees to diseases or pesticides likely accelerates bee colony collapse (Pasquale et al., 2013).

3. AIMS OF THE STUDY

In order to prevent or diminish bee decline, it is at first essential to fill gaps in knowledge about the precise distribution of pesticide residues in the environment, their relationship with forage plants, and their spread among honey bee colony components. Knowing the spread of chemicals belonging to different chemical classes among honey bee colony components helps to design more optimal beekeeping practicides. Special attention must be paid to lethal and sub-lethal effects of chemical mixtures on different bee species and developmental stages. As an alternative to synthetic pesticides, microbiological preparations are used in plant protection. However, little is known regarding their effects on bees. The objectives of this work included:

1. To determine different pesticide residues from honey bee colony components in different landscapes, and to test whether there is a correlation between the proportion of cultivated oilseed rape and pesticide residues found (**Paper I, II**).

   H1: There are differences in bee matrices, regarding the content of pesticide residues.

   H2: Oilseed rape is a potential source of pesticide contamination, but honey bee preference to it is low due to species richness of flowering plants in Estonia.

2. To investigate how two different lipophilic pesticides and their mixtures in honey bee wax are affecting honey bee queen development (**III**).

   H3: Exposure to field relevant concentrations of two different pesticides and their mixtures can negatively affect honey bee queen development and mating .

3. To study whether there are synergistic effects on bumble bee mortality and feeding rate between an EBI fungicide and four different insecticides, which are representing major chemical families used in farmland crop protection (**IV**).

H4: Exposure to the EBI fungicide imazalil increases insecticides (fipronil, thiamethoxam, imidacloprid and cypermethrin) toxicity, and thus bumble bee mortality increases significantly.

4. To investigate three microbiological preparations impact on honey bee and bumble bee longevity and metabolic- and water loss rate (**V**)

   H5: Microbiological preparations have different effects on honey bee and bumble bee longevity and metabolic- and water loss rate.

4. MATERIAL AND METHODS

4.1. Field data collection

4.1.1. Study areas and materials analyzed

The samples of bee and bee products for pesticide residue analysis were collected in 2013 and 2014 from apiaries located in Eastern and Southern Estonia (**I, II)**. Twenty-three apiary sites from Eastern-Southern part of Estonia were used in years 2013 and 2014. Honey bee colony components collected from each apiary for pesticide analysis were as follows: brood, nurse bees, beebread, corbicular pollen (**I**) and honey (**II**). In two consecutive years, 140 samples were collected in total. Samples were collected either once or twice per year according to blooming of spring or winter oilseed rape. The landscapes around the selected apiaries represented variable land use types from 70% of agricultural land to almost 100% forested areas. Using Arc-GIS (version 10.1, Esri, Redlands, CA, U.S.) the land use type was calculated. In calculations, both 2 or 4 km radii from each apiary were added to landscape analyses (**I, II**).

4.1.2. Pesticide selection for residue analyses

According to the Tartu County Farmers Association´s pesticide ordering list, the 47 most common (based on quantities) active ingredients were chosen for pesticide residues analysis from honey bee colony components in 2013 and 2014. There were 21 herbicides, 15 fungicides, 10 insecticides and one plant growth regulator among the tested pesticides (**I**, **II**).

4.1.3. Determination of botanical origin of pollen and honey

Pollen was collected at the entrance of the hive by using pollen traps in order to prevent in-hive contamination by pesticides. Pollen sampling was made during the flowering of both winter and spring oilseed rape. Honey was collected right after the flowering of spring oilseed rape. The botanical origin was determined in the laboratory using light microscopy (400x magnification) (Olympus CX 31 RBSF), using the standard acetolysis method (**I**). Pollen grains were identified by comparison to a reference pollen collection and the relevant literature (**I**).

4.2. Toxicity experiments

4.2.1. Origin of materials

In the experiment where honey bee queens were exposed to two different pesticides and their mixtures, the queens used originated from the local company OÜ R-honey (**III**). Bumble bee (*B. terrestris*) colonies used in the fungicide-insecticide synergy study originated from companies Koppert Biological Systems (Berkel en Rodenrijs, Netherlands) and Biobest (Westerlo, Belgium), and the toxicology studies were performed in laboratories at Exeter University and the Estonian University of Life Sciences (**IV**). In the microbiological preparation experiment, the honey bees used originated from the local company OÜ R-honey, and bumble bees were purchased from Koppert Biological Systems. The experiments were conducted at the Estonian University of Life Sciences (**V**).

4.2.2. Larval development of honey bee queens

To investigate toxicity of wax-dissolved pesticides on developing honey bee queens, an experiment was conducted in two consecutive years (2017 and 2018). Field realistic concentrations of the fungicide tebuconazole (412 μg kg^{-1}) and acaricide tau-fluvalinate (15 μg kg^{-1} and 446 μg kg^{-1}), and their mixtures, were mixed into molten organic wax, and subsequently the queen cell cups were made using special wooden dowels. The tau-fluvalinate concentrations used in the experiment remain within the range of residues found from Estonian wax samples collected in 2013/2014 (Raimets unpublished data). The tebuconazole concentration used was higher than found from Estonian wax (Raimets unpublished data), but still field realistic according to findings from other bee products (**I**). Two different concentrations of tau-fluvalinate were used in the experiment, due to large variation of tau-fluvalinate concentrations found from bee products. The parameters measured were: queen cell acceptance, hatching, newly emerged queen weight, and mating. In both experimental years, one-day-old honey bee larvae from a single colony were grafted into previously performed queen cells. Grafted cells were placed into queenless colonies full of nurse bees, and 24 h later it was monitored whether the bees have started feeding the larvae or not. On the 5th day, accepted and sealed cells were placed into an incubator where the ambient temperature was a constant 34.5°C and RH 60%. On the 10th day, the sealed cells were caged, and two days later the queens hatched. All the hatched

queens were weighed and then inserted to a mini 4-frame nucleus colonies, which were then located to special mating yard. After 2 weeks each mini-hive was controlled for queen onset of oviposition (**III**).

4.2.3. Bumble bee toxicity

To test synergistic effects of pesticides on wild bees, another experiment was conducted. The impact of single pesticides and their mixtures on bumble bee mortality and feeding rate were measured. Different combinations of the fungicide imazalil (300 mg L^{-1}) and four insecticides (fipronil (20 µg L^{-1}), thiamethoxam (13 µg L^{-1}), imidacloprid (500 µg L^{-1}) and cypermethrin (7 mg L^{-1}) were mixed into syrup (Attraker, Biological Systems, Berkel en Rodenrijs, Netherlands). All chemicals used were purchased from Sigma Aldrich. Bumble bee workers from queenright colonies were individually and randomly allocated into small wooden cages equipped with eppendorf tubes containing distilled water and sugar syrup (Attraker, Biological Systems, Berkel en Rodenrijs, Netherlands). Bees had constant access to food during the experiment. Formed mini hives (7 x 5 x 4 cm) with bees were kept in a semi-controlled environment (24±1°C, ~47% relative humidity, 12:12 h dim light:darkness). Bumble bee mortality was recorded after 24 h and 48 h. Feeders were weighed before the experiment and after 48 h, in order to determine syrup consumption (**IV**).

4.3. Testing microbial pesticides on honey bees and bumble bees

Effects of microbiological preparations and some other carrier compounds on honey bee and bumble bee mortality and physiology were measured. The biofungicide Prestop-Mix (spores of *Gliocladium catenulatum*) was obtained from the company Verdera (Espoo, Finland). The bioinsecticides BotaniGard (spores of *Beauveria bassiana*) and Met52 (*Metarhizium brunneum*) were purchased from Borregaard BioPlant ApS (Aarhus, Denmark). In addition, effects of pure *G. catenulatum* spores (obtained from Verdera) on bees were also tested. Impact of kaolin and wheat flour as carrier compounds were also monitored. Forager honey bees were collected from a single queenright colony, and worker bumble bees were collected at the entrance of the two hives. Each honey bee was treated individually by any of the treatments used. Honey bees used were gently shaken in vials containing 20 mg of each treatment Bumble bees were also individually treated with 50 mg of each of the treatment. Control group bees were shaken gently in an empty vial.

In the survival experiment, both bees species previously exposed to any of the treatments used were transferred to wooden mini hives (7 x 5 x 4 cm; both 7 cm openings were covered by wire mesh). Each mini hive consisted of 20 worker honey bees or one bumble bee forager. All the mini hives were equipped with sugar syrup and distilled water tubes. Performed mini hives were located into an incubator (SANYO - Versatile Environmental Test Chamber, MLR-351, Japan), where ambient temperature was constantly 28°C, RH 60% and light regime 12:12 light:darkness. Bee survival was daily monitored until all bees were considered dead.

Effects of previously mentioned treatments on both bee species metabolic- and water loss rate were also investigated. Metabolic rate (MR VCO_2 ml h^{-1}) and water loss rate (WLR VH_2O µl h^{-1}) were measured using an LI-7000 flow-through respirometer (LiCor, Lincoln, NE, U.S.). In addition to respiratory water segregation, cuticular water loss in insects has also been observed. Thus, it is essential to investigate whether, and to which extent, the treatments used affect insect total water loss rate. To obtain reliable results, magnesium perchlorate ($Mg(ClO_4)_2$) and potassium hydroxide (KOH) were used to remove superfluous water and carbon dioxide from air entering the system. Each bee spent 3 h in the respirometer both before and after treatments (**V**).

4.4. Chemical analyses of pesticide residues

Pesticide residues in all bee matrices and in honey bee queen pupae were analyzed at the Institute of Food Safety, Animal Health and Environment "BIOR" (Riga, Latvia). For most compounds tested, the QuEChERS extraction methodology was used, followed by detection using gas chromatography-mass spectrometry (GC-MS) and ultra-high performance liquid chromatography tandem mass spectometry (UHPLC-MS/MS). In the case of glyphosate, aminopyralid and clopyralid, UHPLC-MS/MS was performed as a single analysis (**I**, **II, III**).

4.5. Data analysis

Software Statsoft (version 12, USA) was used to analyse the data (**I, II**). A χ^2 test was used to collate the number of pesticides found and searched from collected samples. To evaluate the effects of sampling year and period in different matrices, the Kruskal-Wallis H index was used. The

quantities of pesticide residues in honey bee colony components were tested by using Wald χ^2 test. ANOVA was used to compare the proportions of *Brassica napus* in pollen samples. Statistical software R (3.5.1.) was used to assess the significance of associations between:

a) cultivated and forested land, and cultivated land within a 2 km and 4 km radius (**I**);
b) total sum of residues vs sum of pesticides from different classes (insecticides, fungicides, herbicides) (**I, II**);
c) diversity of plant taxa in different land use types where honey bee foragers were foraging (**I, II**);
d) pesticide residues and bee collected pollen from different plant taxa (**I**);
e) pesticide residues and honey collected from different plant taxa (**II**); and
f) pesticide residues and proportion of land use type within a 2 km radius around each hive.

To test the effects of two pesticides and their mixtures on honey bee queens, the program STATISTICA (version 13) was used. Two-way full factorial analysis of variance (ANOVA) with *post-hoc* Tukey test was used to determine the treatment effects on emerged queen weight. To assess each treatment's impact on queen cell acceptance, a χ^2 test was used. The results were considered statistically significant when $p < 0.05$ (**III**).

To test synergistic interactions between the fungicide and a single insecticide, the modified binomial proportion test for additivity (BPA) was used. For each of the four insecticides, BPA tests were used separately for 24 h and 48 h mortality. To determine the variation in feeding rates among treatment groups, a one-way analysis of variance (ANOVA) with *post-hoc* Tukey test was used. The amount of food consumed was measured only after 48 h (**IV**).

ANOVA and Kruskal-Wallis were used to test microbiological preparations and other carrier compounds' impact on honey bee and bumble bee survival. To test whether there were statistically significant differences in both bee species' metabloic- and water loss rate among different treatment groups, a factorial ANOVA was used. To assess the differences in metabolic- and water loss rate pre- and post-treatment, a paired t-test was performed (**V**).

5. RESULTS

5.1. Pesticide residues in beehives

5.1.1. Occurrence in different matrices

The results showed that, from the 140 samples collected, 17 different pesticide residues from all three basic pesticide classes were found from honey bee colony components. Interestingly, the proportion of pesticides from different chemical classes did not reflect the actual number of residues found. 80% of pesticide residues detected were insecticides, followed by herbicides (24%) and fungicides (27%) (χ^2=81.96; df=2; $p < 0.001$; Figure 4, **I**). Tau-fluvalinate was the most commonly detected compound in the collected samples (found in 39 samples), followed by thiacloprid (36) and tebuconazole (22) (Table 1, **I**).

The number of detected pesticide residues varied significantly between different honey bee colony components (Wald $\chi^2(4)$=9.671; p=0.046). The majority of pollen samples (96.6%) were contaminated by 1 – 6 different pesticides (**I**). 95.5% of beebread samples were contaminated with 1-8 different chemicals (**I**). Insecticide and fungicide residues were most frequently found in pollen and beebread samples (Table 1, **I**). 69.6% of honey samples contained 1 - 3 pesticidal compounds, and herbicide residues were the most frequently detected from samples (Figure 4, **I**; Table 3, **II**). 61% of nurse bee samples contained only a single pesticidal compound per sample, and herbicide residues were dominant (Table 1, **I**). Brood was less contaminated (43.6% of samples) than pollen or beebread; only 1 - 2 residues per sample were detected in brood, and no correlation between brood and specific chemical class was found (Table 1, **I**).

Results also revealed that there was a significant difference between sampling year and number of residues found. In 2014, pollen and beebread samples contained more pesticide residues than in 2013 (Table 2, **I**). In contrast, honey samples contained significantly more residues in 2013 (Figure 1, **II**). There was no correlation between sampling year and residues found in brood (Table 2, **I**).

5.1.2. Botanical origin of collected pollen and honey

When analysing the botanical origin of pollen, the results showed that among all gathered pollen, oilseed rape pollen represented 51.9%. In July, during spring oilseed rape flowering, bees collected more cruciferous crops pollen (71.8%) than in May (41.9%), where in May winter oilseed rape was flowering ($F(1;25)=6.95$; $p=0.014$) (**I**). Plant taxa detected from pollen differed with sample collection time. In May, the Brassicaceae were dominant among all gathered pollens (56.1%), followed by Rosaceae (30.53%) and Fabaceae (6.53%). In July, the bees plant species preferences were more diverse, however the Brassicaceae comprised 73.28% of collected pollen, followed by Fabaceae (12.65%), Rosaceae (6.5%) and Apiceae (5.59%) (**I**).

Plant taxa detected in honey samples were more diverse. The most abundant pollen grains found in honey samples collected were that of oilseed rape (25.9%). The most frequent families detected in honey were: Brassicaceae (39.4%), Fabaceae (19.6%), Rosaceae (15.5%), Salicaceae (8.9%), Apiaceae (5.6%) and Asteraceae (4.9%) (**I**). The polyfloral origin of the honey can be explained in that most Estonian beekeepers harvest honey only once in august, when the main honey flow ends.

There were no significant correlations between land use type and plant taxa in pollen samples. Interestingly, the presence of *B. napus* in pollen samples was not significantly correlated with land use type, even when the 2 km around-hive radius was expanded to 4 km (Figure 3A, B, **I**). Nevertheless, in May some plant taxa in pollen was directly associated with certain land use characteristics, and in July there was a negative correlation between Fabacea pollen and cultivated land within a 4 km radius.

There was a strong correlation between the proportion of *B. napus* pollen in honey and the percentage of cultivated land within both 2 km and 4 km radii (Figure 3C, **I**). There was also a positive correlation between cultivated land and other cruciferous plants.

5.1.3. Correlations between plant taxa and residues detected

There were no significant correlations between pesticide residues found in samples and different land use characteristics (all correlations $r \leq 0.15$)

(**I**). In May, significant negative correlations between percentage of oilseed rape pollen found and the amount of both alfa-cypermethrin and cypermethrin were observed (Figure 4A, **I**). Still, there was a significant positive correlation between dimethoate residues detected in *B. napus* and Apiaceae pollen in May (Figure 4A, **I**). A positive correlation between thiacloprid residues in Apiaceae was also observed (Figure 4A, **I**). In general, the total sum of pesticides detected in May showed negative correlation with oilseed rape.

In July, there was only one significant correlation found between the botancial origin of pollen and an pesticidal compound. Residues of thiacloprid were significantly correlated with Apiaceae (Figure 4B, **I**). Positive trends were also observed between the presence of insecticides alpha-cypermethrin and cypermethrin and the herbicide MCPA in oilseed rape pollen collected in July. In addition, positive correlations were also observed between Fabaceae pollen and prothiconazole, tau-fluvalinate and thiacloprid (Figure 4B, **I**).

There were less positive correlations between pesticide residues detected in honey and plant taxa. In general, only herbicide residues were positively correlated with non-crop Brassicaceae (Figure 4C, **I),** and herbicide residues were most commonly found from honey samples (Table 3, **II**). Nevertheless, there were strong correlations between thiacloprid residues and Fabaceae, as well as between tau-fluvalinate residues and Apiaceae in honey samples (Figure 4C, **I**).

5.2. Toxicity of chemical pesticides

5.2.1. Wax treatment on honey bee queen development

Results showed that only tau-fluvalinate residues were found in honey bee queen pupae in the tebuconazole treatment group (Table 1, **III**). Results also showed that, in 2017, queen cell acceptance did not differ significantly between treatment groups ($\chi^2 > 0.441$); p=0.931). However, in 2018 queen cell acceptance was significantly lower in the tebuconazole treatment group ($\chi^2 > 24.378$); p=0.001) (Figure 1, **III**). No synergistic effects regarding queen cell acceptance were observed in either year.

Both tebuconazole and the lower concentration of tau-fluvalinate increased newly emerged honey bee queen weight significanlty in 2017

(F(3;43)=4.99; p=0.005), but no synergistic effect was observed (Figure 2, **III**). In contrast, in 2018 tebuconazole had no impact on newly hatched queen weight (Figure 2, **III**). The tebuconazole concentration used was that which was detected from bee brood in Estonia (**I**); as a lipophilic compound, it has ability to spread into wax. In 2018, the higher tau-fluvalinate concentration used increased emerged honey bee queen weight significantly (F3;57)=3.5674; p=0.019), but no synergistic effect was observed (Figure 2, **III**). There was no significant difference in emerged queen weight between the two tau-fluvalinate treatments (χ^2=0.40; df=1; p=0.53). Both tau-fluvalinate concentrations used were in the range of concentrations found from wax collected in Estonia in 2013. In those samples the concentrations ranged between nearly 0 and 518 μg kg^{-1} (Raimets unpublished data). The highest concentration found from wax was even higher than that which was used in the present study (**III**). When comparing emerged honey bee queen weight between the two experimental years, results show that, in the first year, queens weighed significantly more (F(1,106)=38.201; p=0.001). The pesticide concentrations used did not affect queen hatching, and there were no differences in queen mating in both experimental years.

A significant interaction effect between tau-fluvalinate and tebuconazole on queens weight was observed in 2017 (F(1,43)=11.87, p=0.001). A similar tendency was observed in 2018, although the interaction effect was not statistically significant (F(1,57)=3.32, p=0.074) (**III**).

5.2.2. Oral treatment on bumble bee workers

No signifcant effect on bumble mortality occurred when bees consumed non-treated syrup (control group). The effects of single pesticides and their mixtures on bumble bee mortality and feeding rate are shown in table 2.

Bumble bees that consumed syrup mixed with the fungicide imazalil and the insecticide fipronil exhibited significantly higher mortality after 24 h, but not after 48 h (24 h: BPA test, p<0.005; 48 h, n.s.) (Figure 1, 2, **IV**). Also, imazalil and the neonicotinoid thiamethoxam, when combined, resulted in a synergistic toxicity, increasing bumble bee mortality significantly after 24 h and 48 h (24 h, 48 h: BPA test, p<0.005) (Figure 1, 2, **IV**). Synergistic interactions were also observed from combined dietary exposure to imazalil and cypermethrin, where bee mortality inc-

Mortality of bumble bee					Bumble bee feeding rate	
	Single pesticide effect		Synergistic effect with imazalil		Single pesticide effect	Synergistic effect with imazalil
Pesticide	After 24 h	After 48 h	After 24 h	After 48 h	After 48 h	After 48 h
Imazalil (300 mg L^{-1})	Yes	Yes	-	-	Yes	-
Fipronil (20 µg L^{-1})	No	Yes	Yes	No	Yes	No
Thiamethoxam (13 µg L^{-1})	No	No	Yes	Yes	No	No
Imidacloprid (500 µg L^{-1})	No	No	No	No	No	No
Cypermethrin (7 mg L^{-1})	No	No	Yes	Yes	Yes	No

Table 2. Synergistic effects of single pesticides and their mixtures on bumble bee mortality and feeding rate.

reased significantly at both 24 and 48 h (24 h, 48 h: $p<0.001$) (Figure 1, 2, **IV**). Exposure to imidacloprid alone increased bee mortality slightly. Nevertheless, despite the quite high used concentrations, combining imidacloprid and imazalil did not result in any synergistic effects on bumble bee mortality (Figure 1, 2, **IV**).

Our results showed that exposure to different pesticides caused variation among bumble bee feeding rates (one-way ANOVA, fipronil: $F_{3, 87} = 17.1$, $p < 0.001$; thiamethoxam: $F(3,60) = 15.6$, $p<0.001$; imidacloprid: $F(3,73) = 5.2$, $p<0.01$; cypermethrin: $F(3,64) = 25.3$, $p<0.001$), and in general syrup consumption decreased (Tukey *post-hoc* tests, $P \leq 0.05$). Despite the single chemicals' impact on bumble bee feeding rate, no synergistic effects were observed (Figure 3, **IV**).

5.3. Effects of microbial pesticides on honey bees and bumble bees

When survival was monitored as the response variable, bumble bees lived significantly longer than honey bees (KW-$H(1;80)=44.9$; $p<0.001$) (**V**). Different powdered treatments significantly affected the longevity of both bee species (bumble bees: Kruskal-Wallis$(4;97)=16.2$; $p<0.01$; honey bees: KW-$H(6;480)=152.9$; $p<0.001$). Non-treated bees and bees

treated with wheat flour lived significantly longer than other treatment groups. Interestingly, the biofungicide Prestop-Mix significantly decreased honey bee survival. However, pure *G. catenulatum* did not affect honey bee survival. Surprisingly, pure kaolin also had similarly to used bioinsecticides significant negative impact on both bee species longevity (Figure 1, **V**).

Both measured physiological parameters MR and WLR, were significantly lower in bumble bees compared to honey bees (MR: $F(1;64)=3.9$; $p=0.05$; WLR: $F(1;64)=24.7$; $p<0.001$). MR and WLR did not decrease with time in honey bees (MR: $t= -0.37$, $df=3$, $p=0.74$) (WLR: $t=0.68$, $df=3$, $p=0.55$). However, MR decreased significantly in bumble bees ($t=7.18$, $df=5$, $p<0.001$). Time after start of treatment did not result in any changes in bumble bee WLR ($t=1.36$, $df=5$, $p=0.23$) (**V**).

None of the biopreparations, nor other powders usedhave any substantial effect on both bee species' MR (VCO_2 ml h^{-1}) ($F(4,42)=0.32$, $p=0.86$). The observed variation in MR was greater among honey bees than for bumble bees ($F(1,42)=7.39$, $p=0.009$) (**V**).

In contrary to MR, the treatments significantly affected both bee species' WLR (VH2O µl h-1) (honey bee: $F(6,29)=35.54$; $p<0.001$; bumble bee: $F(4,20)=6.75$; $p=0.001$). Prestop-Mix and kaolin significantly increased both bee species' WLR. BotaniGard significantly decreased WLR in honey bees, whereas Met52, *G. catenulatum* spores and pure wheat flour did not (Figure 2, **V**).

6. DISCUSSION

6.1. Pesticide residues in bees and beehives, relationship with surrounding landscape and forage plant species

Pesticide residues were detected from different honey bee colony components, which were collected from hives located within various land use types. We also investigated the specific botanical origin of pesticides in order to disprove some common wrong understanding in society that all pesticide residues are associated only one certain crop (**I, II**).

The present work showed that various pesticide residues were found in different bee matrices. However, most of the pesticides detected were present at very low concentrations near the lower limit of detection. The results of our study showed that pollen and beebread samples are mostly contaminated with insecticides, followed by fungicides. In addition, among all pollen collected, oilseed rape pollen represented 51.9%. Though most of the PPP, of which residues were detected from pollen, may be related to oilseed rape production, they are probably used on most other crops as well. This could be why there was no observed correlation between *B. napus* pollen and the number of residues detected from collected pollen (**I**). In May, there are numerous wild flowering plants and flowering crops that bees may visit. There is clear evidence that mass flowering crops can increase bumble bee foraging distances by higher attraction rate (Walther-Hellwig and Frankl, 2000). Honey bee foraging distances have been shown to be up to 5 - 6 km (Beekman and Ratnieks, 2000), although normally they forage within a 2 - 3 km radius. In general, wild pollinator foraging distances are significantly lower than honey bees. Different solitary bee species' foraging distances have been shown to be between 150 - 600 m (Gathmann and Tscharntke, 2002), which does not exclude their exposure to pesticides.

Due to differences in climate and agricultural practices, the proportion of different pesticide residues in bee products may vary significantly between countries. In contrast to the present work, a study conducted in Florida and California apiareis in North Amercia showed that fungicide residues were mostly found in pollen and beebread samples (Mullin et al., 2010), which could be explained by warmer climate and more favourable conditions for fungal pathogens. However, studies conducted in Spain and France show results similar to our work (**I**), in that insec-

ticide residues were most frequently found in pollen (Bernal et al., 2010; Chauzat et al., 2006).

Estonian honey is polyfloral and relatively free of pesticide residues. Low levels of contamination in honey was observed by (Kasiotis et al., 2014). Our results revealed that, among the few pesticides detected in honey samples, the herbicides glyphosate and clopyralid were the most dominant (**I, II**). There were also statistical differences between year and number of residues detected in honey samples. In 2013, honey contained significantly more pesticide residues than in 2014 (**II**). This may be due to variation of weather and the need for plant protection operations. Also, pesticides differ in their chemical composition and physical characteristics, which helps us explain why there are dissimilar pesticides found in different bee matrices. Lipophilic pesticides easily accumulate into wax, whereas water-soluble compounds can be more readily found in honey and nectar. Some common herbicides like glyphosate are water-soluble and may stay in an environment for long periods (Balbuena et al., 2015), which helps us understand why herbicide residues were most prevalent in honey samples (**I, II**).

Unlike findings for pollen, honey samples were present in a more diverse array of plant species (**I**). This may be due to the fact that beekeepers in Estonia harvest honey principally only in August, and thus flowering plants blooming from spring to late summer can be found in honey. However, oilseed rape pollen was the most prevalent in honey (25.9%) samples collected (**I**). Despite rich and diverse foraging sources during the main honey flow in July, bees still seemed to prefer oilseed rape fields, likely due to the abundance of flowers, even if they had to fly longer distances (**I, II**). There was no statistically significant correlation between the proportion of oilseed rape and the number of pesticides detected in honey (**I, II**). Some herbicide residues detected in honey may have originated from flowering weeds, since glyphosate is also used to treat weeds along road edges and in other greenery works in horticulture, and thus forager bees visiting flowering weeds may also be exposed to herbicides (Estonian Agricultural Board, 2019).

Despite the fact that pollen, as the main source of bee food, can be relatively highly contaminated with pesticides (David et al., 2016; García-Valcárcel et al., 2019), the bees themselves seem to be less contaminated (Mullin et al., 2010). The results of our study show that only two

herbicides (glyphosate and MCPA) and two insecticides (lambda-cyhalothrin and tau-fluvalinate) were detected in nurse bees, indicating lower contamination rates compared to the number of pesticide residues detected in pollen (**I**). In our study, herbicide concentrations found in bees were much higher than that of insecticides. It can be assumed that most pesticide residues detected in bees originate from agricultural practice; only tau-fluvalinate is used simultaneously in both agriculture and apiculture, and thus the initial source remains unknown (**I**). Fulton et al. (2019) also showed that pesticide residues were less often found in pollen collected in forests. Nevertheless, wild pollinators who live in grasslands may still be exposed to different pesticides. A study conducted in the U.S. focusing on exposure of native bee species to agrochemicals observed that, from native bees collected in grasslands, residues of numerous pesticides were detected (Hladik et al., 2016).

It has been shown that nurse bees can filter out xenobiotic compounds from beebread when producing royal jelly (RJ) for larvae (DeGrandi-Hoffman et al., 2013). While filtering out xenobiotic compounds from beebread, the accumulation of pesticides in nurse bees seems inevitable. Nevertheless, the low pesticide concentrations found in nurse bees, even if the other materials are contaminated (**I**), indicates the process of pesticide detoxification via different enzyme systems in bees.

Similar to nurse bees, sealed honey bee brood was also less contaminated with pesticides. Here, residues of only two insecticides (tau-fluvalinate and dimethoate), one herbicide (glyphosate) and one fungicide (tebuconazole) were found, and in general the concentrations were low (**I**). Tebuconazole was detected in only one brood sample, and thus this cannot be considered to be a reliable indicator of agricultural pollution. In addition to agricultural use, many wood processing companies require fungicides to impregnate their wooden products, and thus it is likely that some fungicide residues detected in bee products may originate from wood industries.

Despite low brood contamination (**I**), developing honey bee larvae remain threatened by pesticide exposure. Several studies show that honey bee wax can be contaminated by various pesticides (Chauzat and Faucon, 2007; Harriet et al., 2017; Manning, 2018; Ravoet et al., 2015). However, another study showed that different field relevant pesticide concentrations in wax had negligible effects on honey bee colony growth and survival. Rather, *Varroa* infestation rate was suggested as the decisive

factor of honey bee colony wintering success (Payne et al., 2019). Lipophilic pesticides can easily accumulate into wax (Ravoet et al., 2015) and thus *Varroa* mites that are mainly feeding on sealed brood are constantly exposed to these chemicals, which in turn may favor the development of resistance. An Argentinan study showed the relationship between coumaphos residues in wax and the potential development of resistance, using the resistance index (RI). There was a positive relationship between residues in wax and RI (Medici et al., 2015). These results suggest that even if *Varroa* treatment applied in strategic rotation, external pollution may still result in the development of resistance in *Varroa* mites, leaving honey bees more vulnerable due to the lack of resistance development.

6.2. The influence of pesticides in wax to honey bee queens

The effect of pesticides on developing honey bee queens was investigated via contaminating wax with different concentrations of pesticides and their mixtures (**III**). Previously, only insecticidal effects on honey bee queens were measured, but it is also vital to investigate other pesticides like fungicides, alone and in combination with other pesticides.

Results of our work showed that low concentrations of pesticides in wax had a significant impact on certain parameters of developing honey bee queens (**III**), although no detrimental effect was detected. Tebuconazole significantly decreased queen cell acceptance by nurse bees in 2018. However, in 2017, no negative effects were seen. Tebuconazole-treated and non-treated (control) queen cell cups were attached to the same langstroth frame and inserted into same queenless colony, excluding the possibility that bees randomly rejected tebuconazole-treated cups.

The impact of pesticides on bees can be influenced also by the current weather and surrounding climate. Colin et al., (2019) performed identically designed studies in Australia and U.S. and they found that imidacloprid had significantly different effects on honey bee colonies in two different continents, which may be linked to different climatic and environmental conditions. In the present study, differences in queen cell acceptance may also be caused by different weather conditions between two experimental years (**III**).

Starving honey bee larvae produce a pheromone called E-β-ocimene to signal nurse bees to feed them (He et al., 2016). There is a possibility

that tebuconazole affects a developing queen larva's hormonal system, which may interfere with pheromone production, and thus nurse bees may not receive the signal to initiate feeding. There is no clear explanation on why tebuconazole had a significant effect on cell acceptance only in 2018. However, in general, the queen bees weighed significantly less in 2018, which may support the idea that, due to poor nutrition, the bees were forced to produce less RJ for queens, making tebuconazole contamination an extra stress factor amplifying the negative effect of nutritional stress. De Souza et al. (2019) showed that developing queens receiving more diverse food (combination of sugar-enriched RJ and juvenile hormone) had more viable sperm and higher number of sperm count. They also found that grafted first instar larvae developed into higher reproductive quality queens than third instar larvae.

Tebuconazole and tau-fluvalinate had significant negative effects on newly emerged honey bee queen weight (**III**). Similar to cell acceptance, tebuconazole had a significant effect on queen weight only in one experimental year (2017). However, this effect was not relevant, and no synergistic effect on queen weight was observed in either experimental year. Tebuconazole is a lipophilic compound that easily absorbs into wax (García et al., 2017); due to continuous agricultural application, concentrations of its residues in wax will increase over time, and thus it is a matter of time until negative effects on bees may appear. Interestingly, we saw rather antagonistic effect of tebuconazole and tau-fluvalinate mixture on queen weight on both experimental year and thus there were no significant differences in queen weights between non-treated and queens exposed to pesticide mixtures, however tau-fluvalinate as a single compound significantly increased newly emerged honey bee queen weight in both experimental years (**III**). Tau-fluvalinate concentrations differed almost 30 times between the two years. Our results are contrary to those of other studies, where exposure to acaricides led to significant decreases in hatched queen weight (Haarmann et al., 2002; Pettis et al., 2004). Thiamethoxam has also been shown to significantly decrease emerging honey bee queen weight (Gajger et al., 2017).

We propose that, in our study, the tau-fluvalinate concentrations in wax affected the hormonal system of developing queen larvae. The corpora allata is a vital gland located in the bee brain. It is responsible for juvenile hormone production (Medici et al., 2012), and thus helps to maintain insect homeostasis. There is the possibility that tau-fluvalinate negatively

affected the queens' hormonal system, resulting in increased food consumption and in turn queen weight (**III**). We suggest that tebuconazole had an antagonistic effect when in mixture with tau-fluvalinate, as tau-fluvalinate as a single compound, in both years, significantly elevated emerged queen weight; whereas in combination with tebuconazole, no significant effect on queen weight was observed.

These observed changes in queen weight cannot be detected by the human eye, and thus this complicates the issue. However, one study showed that there was no significant correlation between newly emerged queen weight and her subsequent behaviours such as time from emerging to mating and time from emergence to oviposition (Kahya et al., 2008). Nevertheless, it has been shown that the number of drones with whom honey bee queens are mating is a critical factor for subsequent colony development due to the varying genetic make-up of drones (Chapman et al., 2019). Moreover, a recent study showed that the most common acaricides and fungicide, and their mixtures, in honey bee wax did not have any significant effects on the amount of bee brood, number of adult bees, or colony growth in general (Payne et al., 2019). It is unlikely that pesticides in wax do not favor bee fitness in general, and there is always the persistent threat of simultaneous exposure pesticides alongside any other stress factor (e.g. diseases, *Varroa*).

6.3. Effects of pesticide mixtures on bumble bee mortality and feeding rate

Effects of four different insecticides, including their mixture with a fungicide, on bumble bee (*B. terrestris*) mortality and feeding rate was observed (**IV**). Most studies have focused on the effects of single pesticides on bees. However, farmers often tank-mix agrochemicals for field application, and thus it is important to determine the effects of pesticide mixtures on bees.

The results of our study showed that three out of four insecticides used showed synergistic toxicity when combined with the fungicide, and this led to increased bumble bee mortality. Bumble bees fed with the insecticide fipronil and the fungicide imazalil in mixture showed significantly higher mortality after 24 h. This suggests that imazalil inhibited the synthesis of cytochrome P450 monooxygenases in the bumble bees, and thus the toxicity of fipronil became more fatal. Interestingly, no syner-

gistic toxicity between fipronil and imazalil was detected at 48 h. We suggest that imazalil inhibited fipronil detoxification, thus preventing the formation of fipronil sulfone, a metabolite of fipronil that has been shown to be highly toxic to different insects (Hainzl et al., 1998) (**IV**). In contrast, thiamethoxam showed synergised toxicity, at both 24 and 48 h in our study, when combined with imazalil (**IV**), where bumble bee mortality increased significantly. Oral toxicity of thiamethoxam in honey bees has been shown to increase when combined with the EBI fungicide tebuconazole (Thompson et al., 2014). Thiamethoxam has been shown to be more toxic to honey bees than clothianidin (Laurino et al., 2013), a metabolite of thiamethoxam (Simon-Delso et al., 2015). An experiment conducted in southern Sweden shows that honey bee colonies placed near oilseed rape fields sowed with clothianidin-coated seeds did not elicit any negative patterns in colony behaviour or development (Osterman et al., 2019). In our study, we suggest that the formation of clothianidin, via metabolizing thiamethoxam, was disrupted due to imazalil's inhibition of cytochrome P450 enzymes, and this could be one potential driver for increased bumble bee mortality (**IV**). Another neonicotinoid, acetamiprid, and the EBI fungicide propiconazole, when combined, resulted in significant synergistic decreases in honey bee (*A. cerana*) longevity and body weight (Han et al., 2019). Another study shows that propiconazole also synergised and led to increased mortality when combined with the novel butonile insecticide flupyradifurone, a nicotinic acetylcholine receptor (nAChR) agonist (Tosi and Nieh., 2019). Insecticide-fungicide mixtures do not only have negative effect on bee longevity but also to other crucial parameters. One study found that honey bees exposed to different fungicide-insecticide mixtures lived longer, but that their foraging activity was significantly lower, and their energetic metabolism was disrupted (Prado et al., 2019). Nevertheless, these latter studies focused on effects on adult bees, but bee larvae may also be negatively affected by pesticide mixtures. Honey bee larva survival decreased significantly when fed with RJ mixed with different insecticide-fungicide combinations (Wade et al., 2019).

As with thiamethoxam, cypermethrin showed synergised toxicity when combined with imazalil, and bumble bee was significantly at both 24 and 48 h (**IV**). Besides increased mortality, the cypermethrin has been shown to affect the insect nervous system and respiratory patterns (Kadala et al., 2014; Mänd and Karise, 2015; Muljar et al., 2012). In addition, a study clearly indicates that there is a significant increase in toxicity in

honey bees when combining alpha-cypermethrin with the EBI fungicide prochloraz (Thompson and Wilkins, 2003). In our study, we suggest that imazalil disrupted the functioning of detoxification enzymes in bumble bees, and thus the toxicity of cypermethrin resulted in increased mortality, confirming that certain pesticide mixtures are more hazardous to bees than these substances alone.

Although all insecticide concentrations used in our experiment were above the level of field relevance, synergistic effects were not seen between imidacloprid and imazalil (**IV**). There are several works that support our finding that imidacloprid does not synergise with EBI fungicides (Iwasa et al., 2004; Thompson et al., 2014). In honey bees, imidaclopird has been shown to be quickly metabolized (within 24 h) into 5-hydroxy imidacloprid and olefin, which are more toxic to insects than the parent compound (Suchail et al., 2004). While considering the biotransformation kinetics of imidacloprid, it may be possible that bees do not use cytochrome P450 monooxygenases for detoxification, as no increased mortality in the mixture treatment group was observed after either 24 or 48 h. We used high imidacloprid concentration, so metabolization into 5-hydroxy imidacloprid and olefin should have caused some increased mortality in bumble bees.

The pesticide mixtures used did not show any synergistic effect on bumble bee feeding rate at 48 h (**IV**). Although both fipronil and cypermethrin significantly decreased bumble bee feeding rate, no synergistic effect was seen when combined with imazalil. In our study, thiamethoxam did affect the bumble bees' syrup consumption. However, it has been shown that field realistic concentrations of thiamethoxam can negatively affect bumble bee (*B. terrestris*) feeding rate and colony growth (Elston et al., 2013). In our study, we used even higher concentration than (Elston et al., 2013), and we did not see any significant decrease in syrup consumption, though in the mixture treatment the feeding rate decreased significantly. Metastudies regarding the effects of neonicotinoids, across different studies and conditions, are required in order to clarify their potential impact on bees.

6.4. Microbiological preparations impact on honey bee and bumble bee physiology and longevity

Effects of different microbiological preparations and other carrier compounds on honey bee and bumble bee longevity and physiological par-

ameters were measured (**V**). Microbiological powders represent potential alternatives to synthetic pesticides. However, first it is important to determine the effects of these powders on transport vectors (e.g. bees).

The compounds studied had significant negative effects on both honey bee and bumble bee longevity. Prestop-Mix significantly decreased honey bee survival, while pure *G. catenulatum* spores did not. This difference may be due to their different chemical composition, where Prestop-Mix likely contained additives that negatively affect bees. Bumble bees lived significantly longer, and they tolerated the powdered treatments more than the honey bees did (**V**). However, kaolin powder had a significant negative effect on both bee species' longevity. Kaolin has been shown to increase the water permeability of insect cuticle (Golob, 1997). In addition, Karise et al. (2016b) demonstrated kaolin's negative effect on bumble bee longevity. In our study, the bioinsecticide Botanigard also showed a negative effect on both bee species' survival (**V**).

The powders used showed significant effects on both bee species' physiological parameters (**V**). As expected, MR and WLR were significantly lower in bumble bees than in honey bees. Bumble bees as eusocial insects that have the ability to calm down rapidly, and the presence of discontinuous gas exchange (DGE) patterns help to confirm this (Woodman et al., 2008). It has been hypothesized that using DGE helps insects reduce water loss (Schimpf et al., 2009; Vogt and Appel, 2000). Neither the biopreparations used, nor inert materials, elicited significant changes in either bee species' MR (**V**). In contrast, WLR was significantly affected by the powders in both bee species. Kaolin and Prestop-Mix signifcantly increased both species' WLR, but Botanigard increased only honey bee WLR. As in the survival experiment, similar patterns were observed for honey bee WLR when exposed to *G. catenulatum* and Prestop-Mix. Combining *G. catenulatum* spores with some other additives than those found in Prestop-Mix may help mitigate its negative effects on honey bees, allowing sustainable entomovectoring.

As shown by Golob (1997), inert dusts can negatively affect insect WLR via removing the waxy layer of the cuticle and thus causing desiccation. Karise et al. (2016b) showed that kaolin increased cuticular water loss in bumble bees. When considering potential opportunities for mitigating negative effects of powdered biopesticide preparations on bees, it is crucial to first consider the extent to which (taking into account poten-

tial routes-of-exposure) bees are exposed to these powders. In our experiment, the bees were gently coated with powders, and thus there is a possibility that their cuticles were damaged. Using a powder dispenser at the entrance of the hive could possibly help to mitigate the level of exposure, and bee fitness in general being less damaged. As shown by Maccagnani et al. (2005), bumble bees passing the dispenser with overlapping passageways and walked over the micropowder, were all contaminated by the preparation. Nevertheless, there was significantly less powder on flowers carried by insects compared to direct spray treatments, and thus improvements to non-synthetic pest management techniques are urgently needed. Still, Karise et al. (2016a) showed that even a small number of spores per flower can reduce *Botrytis cinerea* Pers infection rate.

6.5. Suggestions for further research

Although bee toxicology studies are as old as pesticide use in agriculture, there are still many knowledge gaps and critical issues to overcome. It is essential to continue investigating the effects of pesticides on bees, as different pesticides can have different effects on various bee parameters; and these effects can vary between different environmental and health conditions. Development of a uniform pesticide risk assessment methodology is vital for synchronizing the work of different scientific groups, and comparisons of data between and among studies relies on this uniformity. While looking for potential reasons for bee decline, it is important to investigate the impact of pesticides and other stress factors in combination. It is vital to consider novel pesticides and pest management techniques in order to develop environmentally friendly agriculture, and these techniques also require rigourous risk assessment.

Bee colony homeostasis can be affected by multiple stress factors. In addition, sublethal effects often cannot be observed by the human eye, which is one reason why we must develop new testing methodologies. For example, using respiratory measurements allows us to detect effects from a different perspective. Another aspect that has gained less attention is the effects of single and multiple pesticide residues in the environment on different developmental stages and castes of bees. Special attention should focus on hazards threatening different bee species, and therefore modifications on research techniques are needed. It is also important to consider the potential threats to bees, regarding non-synthetic pesticides and other alternative plant protection products and technologies.

In order to provide adequate information for farmers, policy makers and other stakeholders, regarding the potential agricultural hazards to both managed and wild bees, ongoing scientific research is essential. Involving novel scientific methodologies and including broader range of taxa into risk assessment will help to find answers for long-term pollinator decline. More inclusive risk assessment, regarding relevant pesticide mixtures, will more accurately reflect the effects of pesticides on pollinator communities.

7. CONCLUSIONS

This work investigated how different pesticide residues, and which concentrations, can be detected in various honey bee colony components collected from different land use types. We observed a correlation between the proportion of oilseed rape in collected pollen and honey and the number of residues detected (**I**, **II**). It also focused on effects of pesticide residues, alone and in combinations, on honey bee queens (**III**) and bumble bees (**IV**), and measured the effect microbiological preparations, as alternatives to synthetic chemicals, on both honey bees and bumble bees (**V**).

This work contributes to understanding how potential stress factors affect honey bee and bumble bee performance. It is important to create connections between bee decline and pesticide residues that occur in the environment.

We showed that there are several different pesticide residues present both in honey bees and bee products (**I**, **II**), and that these were not correlated with landscape parameters or plant species visited (**I**). Likely due to the abundance of flowers in oilseed rape fields, honey bees preferred to fly longer distances, even though there were numerous other plant species flowering nearby. Our results confirmed our hypothesis that there were significant differences, across beehive matrices, in the composition of pesticide residues (**I**, **II**). There were no significant positive correlations between the percentage of oilseed rape and pesticide residues detected in collected pollen. Still, honey bees preferred to forage in oilseed rape fields, especially in July during the main honey flow, and thus our hypothesis was marginally supported.

Field relevant concentrations of pesticides in wax had significant effects on developing honey bee queens. Tebuconazole alone and in mixture with tau-fluvalinate significantly decreased honey bee queen cell acceptance by nurse bees in 2018, however no synergistic effects were observed. Both tebuconazole and tau-fluvalinate singnificantly increased queen weight. Instead of synergy, the antagonism of these two pesticide effects was observed (**III**).

Exposure to fungicide imazalil increased the toxicity of three (fipronil, thiamethoxam and cypermethrin) out of four insecticides in bumb-

le bees, resulting in increased mortality. Imazalil did not synergise the toxicity of imidacloprid, and no bumble bee mortality occurred, indicating the importance of conducting additional research concerning the effects of one of the most widely-used pesticides in the world (**IV**).

Different microbiological preparations have similar effects on honey bee and bumble bee longevity and physiological parameters, however it seems that honey bees tend to be more vulnerable. Due to differences in honey bee and bumble bee physiology and behaviour, the effects of pesticides cannot always be transferred directly from one species to another (**V**).

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SUMMARY IN ESTONIAN

Sünteetiliste ja bioloogiliste pestitsiidide mõjud meemesilastele ja kimalastele

Meemesilaste (*Apis mellifera*) ja ka looduslike tolmeldajate, nagu näiteks kimalaste (*Bombus* spp), arvukus on juba pikemat aega langustrendis ning konkreetset põhjust pole siiani sellele leida suudetud. Esimesed alarmeerivad teated meemesilaste massilisest kadumisest saabusid juba 2006. aastal Ameerika Ühendriikidest, kus esmakordselt täheldati mesilasperede kollapsi sündroomi (*colony collapse disorder;* CCD). CCD tüüpilisemateks sümptomiteks on sööta täis ja mesilaspere poolt hüljatud taru või siis leidub sellises tarus mesilasema koos väga väheste töölismesilastega. Lisaks eelmainitud sümptomitele on täheldatud maailma eri paigus oluliselt sagenevat mesilasperede talvist hukkumist. Seni peeti aktsepteeritavaks talvise hukkumise määraks 10%, kuid tänaseks päevaks on paljud mesinikud erinevates riikides hakanud leppima oluliselt suuremate talvekadudega, mis on suureks ohumärgiks kogu planeedi ökosüsteemile. Lisaks meemesilastele on juba 1980. aastatest alates täheldatud mitme erineva kimalaseliigi arvukuse vähenemist või suisa kadumist nii Ameerikas kui ka Euroopas. Tolmeldajate arvukuse ja liigirikkuse vähenemise põhjusteks on pakutud väga paljude erinevate faktorite koosmõju. Sellisteks faktoriteks on kliimamuutused, monokultuurne põllumajandus, elupaikade fragmenteerumine, haiguste ja parasiitide leviku muutused ning loomulikult pestitsiidide kasutamine.

Paljude erinevate teadustööde tulemused näitavad, et mesilasi ümbritsevas keskkonnas leidub hulganisti erinevate pestitsiidide jääke, mis mõningal juhul paraku kipuvad ka mesindussaadustes akumuleeruma. Ilmekaks näiteks on mesilasvaha, kuhu rasvlahustuvad pestitsiidid aja jooksul kuhjuvad. Samas on vaha nendest puhastada sisuliselt võimatu, mis omakorda seab tugeva löögi alla mesilaste tervise. Probleemi süvendab ka see, et majanduslikult jätkusuutlikus mesilas püütakse vaha maksimaalselt taaskasutada sulatades seda ümber uute kärjepõhjade jaoks, paraku suureneb nii ka vahas leiduvate saasteainete hulk. Väga paljud teadustööd on keskendunud just üksikute pestitsiidide mõjude uurimisele meemesilastel, kimalastel ja ka teistel tolmeldajatel. Mesilased ja mesindussaadused võivad sisaldada korraga mitmetesse erinevatesse klassidesse kuuluvate pestitsiidide jääke. Nii ühe- kui mitmekaupa esinevad

toksilised ühendid omavad kas letaalset või sub-letaalset mõju organismidele, Pahatihti on leitud kogused sedavõrd väikesed, et otsest silmaga märgatavat mõju ei pruugi esineda, kuid see ei tähenda, et need mesilasi kuidagi ei mõjuta. Keerulisemaks muudab situatsiooni veel see, et sama aine sama kogus võib erinevatele liikidele erinevalt mõjuda, Seetõttu on oluline uurida erinevatesse klassi kuuluvate pestitsiidide ja nende segude erinevaid subletaalseid mõjusid kõikidel mesilaste rühmadel.

Lähtudes eelmainitud probleemidest oli käesoleva doktoritöö eesmärkideks:

1. Koguda erineva põllumajandusliku aktiivsusega aladel asuvatest mesitarudest mesilasi ja mesindussaadusi, ning määrata nendes leiduvate pestitsiidide jääkide kogused. Lisaks oli eesmärgiks selgitada välja, kas tarude ümbruses leidunud ja ka mesindussaadustes esinenud rapsi osakaalud ja leitud pestitsiidijääkide hulgad on omavahel seotud (**I**, **II**).
2. Uurida, kuidas vahas leiduvad lipofiilsed pestitsiidid tau-fluvalinaat ja tebukonasool mõjutavad mesilasemade arengut vaglast valmikuni (**III**).
3. Uurida, kas ensüümi P450 biosünteesi takistava (EBI) fungitsiidi ja erinevate insektitsiidide vahel tekib sünergeetiline efekt, mis oluliselt mõjutaks karukimalaste (*Bombus terrestris*) suremust ja toitumist (**IV**).
4. Ning viimaks, uurida, kuidas mõjutavad meemesilaste ja kimalaste suremust ja füsioloogilisi parameetreid sünteetilistele pestitsiididele alternatiivsed mikrobioloogilised preparaadid ja inertsed kandurained (**V**).

Selleks, et teada saada, milliste ja millises kontsentratsioonis pestitsiidide jääke esineb Eesti erineva põllumajandusliku aktiivsusega asuvate alade mesilastes ja mesindussaadustes, koguti kahel järjestikusel aastal proove, mis saadeti keemiliste analüüside tegemiseks laborisse. Kuna rahva seas on levinud arusaam, et pestitsiidid mesindussaadustes pärinevad just rapsilt, siis otsiti antud teadustöö raames ka seoseid proovidest leitud pestitsiidijääkide ja rapsi osakaalu vahel (**I**, **II**).

Uuriti, kuidas mesilaste elukeskkonnast pärinevad ja mesindussaadustes reaalselt leiduvad pestitsiidide jäägid mõjutavad mesilasemade arengut saastunud vaha kaudu. Selleks lisati mahevahale kindlates kontsentratsioonides kahte enim esinenud pestitsiidi ja nende segu ning jälgiti mesilasemade erinevaid arenguetappe vaglast valmikuni (**III**).

Pestitsiidide, ehk siis täpsemalt ühe fungitsiidi segu nelja erineva insektitsiidiga, sünergeetilisi mõjusid uuriti karukimalaste (*B. terrestris*) 24 ja 48 tunni jooksul tarbitava toidu kogust ja suremust jälgides (**IV**).

Kuna sünteetilistele pestitsiididele on hakatud otsima ka alternatiive, siis on oluline esmalt uurida ka nende preparaatide mõju kasulikele putukatele, nagu näiteks tolmeldajatele. Antud töös uuriti kahe erineva bionisektitsiidi, ühe biofungitsiidi ja erinevate pulbriliste kandurainete mõjusid meemesilaste ja kimalaste füsioloogilistele näitajatele ja suremusele (**V**).

Kokkuvõte

Uuringust saadud tulemused aitavad paremini mõista potentsiaalsete stressifaktorite mõju meemesilastele ja kimalastele ning seeläbi luua seoseid nende arvukuse langemise ja keskkonnas leiduvate erinevatest pestitsiidijääkidest tuleneva ohu vahel.

Eesti mesilastest ja mesindussaadustest kogutud proovidest leiti 17 erineva pestitsiidi jääke . Lisaks näitavad tulemused, et enim kasutatud pestitsiidide hulgast leiti eelkõige insektisiidide jääke. Siinkohal on oluline mainida, et kõik insektitsiidide jäägid ei pärine põllumajandusest, vaid ka mõned mesinike poolt varroalesta tõrjel kasutatavad erinevad akaritsiidid sisaldavad sarnaseid aineid. Eesti mesitarudest kogutud proovidest on pestitsiididega enim saastunud just õietolm ja suir. Samas, haudmest ja meest võetud proovidest leiti väga vähesel määral erinevate kemikaalide jälgi, mis loodetavasti annab ka Eestis toodetud mee tarbijatele julgust eelistada just eestimaist. Lisaks lükkavad antud uurimustöö tulemused ümber arusaama nagu pärineks enamik pestitsiidide jääke just rapsi põldudelt. Siiski on oluline rõhutada, rapsi atraktiivsust mesilastele - protsentuaalselt oli just see kultuur enim esindatud nii õietolmu kui ka mee proovides, samas puudus igasugune positiivne korrelatsioon rapsi osakaalude ja leitud pestitsiidijääkide hulkade vahel. Pestitsiidijääke võis mesindussaadustesse koguneda ka näiteks õitsvate umbrohtude esinemisel pritsitavatel põldudel ja maanteepervedel, ning lisaks võisid erinevate kemikaalide jäägid liikuda tuule või pinnasevee abil algsest kasutuskohast eemale teistesse taimedesse, mille õisi mesilased külastasid (**I**, **II**).

Antud doktoritöö tulemustest selgub ka, et vahasse akumuleerunud ja seal püsivad pestitsiidid on potentsiaalselt ohtlikud mesilasemade arengule. Ka Eesti mesindussaadustes sageli esinenud fungitsiidil ja insek-

titsiidil oli oluline mõju mesilasemade vaklade vastuvõtmisele ammmesilaste poolt. Lisaks suurendasid mõlemad üksikud ained koorunud mesilasemade kaalu oluliselt. Uudse faktina tuvastati aga nende ainete omavaheline antagonistlik toime. Otsest mesilasaemade suremust antud katse puhul ei täheldatud, kuid on äärmiselt oluline märkida, et pestitsiidide subletsaalsed mõjud polegi inimsilmale tihti tavalisel vaatlusel nähtavad (**III**) ning seniste andmete põhjal pole täpsemalt võimalik öelda, kuidas suurenenud kaal mõjutab mesilasemade edasist elukäiku.

Lisaks annab antud uurimustöö tunnistust sellele, et erinevate pestitsiidide segud on oluliselt ohtlikumad nende üksikute ainete mõjudest. Tulemustest selgub, et EBI fungitsiid imasaliil omas sünergeetilist toimed kolme insektitsiidiga neljast, mille tulemusena töös kasutatud karukimalastel vähenes tarbitava sööda kogus ning oluliselt suurenes suremus. Niisugune tulemus annab tunnistust sellele, et põllul kasutatavad paagisegud võivad mesilastele ja ka teistele kasulikele putukatele olla väga ohtlikud (**IV**) ning nende kasutuseeskirju tuleb hoolikalt kavandada ja võimalusel segude lubamist üldse vältida. Lisaks on oluline meeles pidada, et kõikide potentsiaalsete segude komplektid tuleb ükshaaval läbi uurida, sest isegi samasse gruppi kuuluvate ainete koosmõjud on erinevad. Antud töö tulemustest nähtub ka, et erinevad mikrobioloogiliste preparaatide ja kandurainete mõjud on meemesilastele ja kimalastele küll mõnevõrra sarnased, kuid väga erineva mõju tugevusega. Erinevate ainete mõju avaldus erinevalt mõlema mesilase liigi suremuses kui ka veekaos. Lisaks ilmnes, et kimalased ja meemesilased on oma olemuselt ja füsioloogiliste näitajate poolest juba sedavõrd erinevad, et teatud juhtudel ei saa ühe liigi katsete tulemusi kanda automaatselt üle teisele (**V**).

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I

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Abstract

Pesticide residues in bee products is still a major issue. However, the relations to botanical source and land use characteristics are not clear. The large variability of residues detected questions the suitability of bee-collected- and other hive materials as indicators for environmental contamination. The aim of our study was to clarify whether different beehive matrices contain similar pesticide residues, and how these are correlated with forage preferences and land use types in foraging areas. We tested bee-collected pollen, beebread, honey, nurse bees and honey bee larvae for the presence of concurrently used agricultural pesticides in Estonia. Samples were collected at the end of May and mid-July to include the main crop in northern region – winter and spring oilseed rape (*Brassica napus*).

We saw that different beehive matrices contained various types of pesticide residues in different proportions: pollen and beebread tended to contain more insecticides and fungicides, whereas herbicides represented the primary contaminant in honey. The variations were related to collection year and time but were not related to crops as basic forage resource nor the land use type. We found few positive correlations between amount of pesticides and proportion of pollen from any particular plant family. None of these correlations were related to any land-use type. We conclude that pesticide residues in different honey bee colony components vary largely in amount and composition. The occurrence rate of pesticide residues was not linked to any particular crop.

Keywords: honey bee, pesticide residues, botanical origin, agricultural landscape, oilseed rape

1. INTRODUCTION

The hazard of pesticides for bees is concerning. Most agricultural practices rely on intensive management strategies, which often include routine or necessity-based spraying of plant protection products. Besides pest control, they also affect development and population sustenance of non-target organisms, decreasing ecological service performance (Helander et al., 2018; Müller, 2018). There is sufficient evidence that the abundance and species richness of pollinators are decreasing, and that pesticides play a partial role in this (Potts et al., 2010; Goulson et al., 2015).

Honey bee (*Apis mellifera* L.) colony development and foraging behaviour make them dependent on superabundant floral resources. In addition to the several wild plants, many agricultural crops represent valuable food resources for honey bees. In northern regions, oilseed rape (*Brassica napus*) and turnip rape (*Brassica rapa*), as mass-flowering crops, represent important floral resources for honey bees (Viik et al., 2012; Requier et al., 2015). However, these crops suffer from several pest insects (Veromann et al., 2012) and thus high pesticide inputs are often applied, which may make the crop even more attractive to pollinators through increasing the number of flowers (Karise et al., 2007). Bringing these two aspects together leads people to believe that oilseed crops in particular are the primary source of contamination in

beehives. However, pesticide drift from agricultural fields to natural areas (Chifflet et al., 2011) can also expose wild plants to pesticides. In addition, exposure to pesticides may occur on flowering weeds inside fields and on field boundaries (Krupke et al., 2012; David et al., 2016).

Modern agricultural practices should lead to decreasing pesticide residue occurrences in nature, an expectation requiring confirmation. Therefore, assessment of environmental contamination requires standardised methods and sampling procedures. For this purpose, people have suggested the use of honey bee products as indicators for environmental contamination because honey bees gather raw material from both wild plants and crops throughout the foraging season, and thus make sampling easier. However, several obstacles may occur. Based on honey samples, for instance, variable contamination is shown by study year (Hladik et al., 2016; Karise et al., 2017) or region, despite the similar agricultural practices (Rodríguez López et al., 2014), and biased findings towards fat-soluble chemicals (Kaczyński, 2017) can occur. Difficulties in explaining the results may arise also due to unknown foraging distances (indicated by presence of pesticide residues in organic honey (Chiesa et al., 2016), forage plants, or even cultivar preferences (Karise et al., 2006). Pesticides themselves can affect the residues detected from hives: altered bee foraging and navigating behaviour (Thompson, 2003; Herbert et al., 2014; Balbuena et al., 2015). Apicultural activity also increases the active compounds (ACs) load in beehives through veterinary treatments of colonies (Bogdanov et al., 1998; Boyle and Sheppard, 2017; Pohorecka et al., 2017).

The aim of our study was to clarify whether different honey bee hive materials reflect similar pesticide residues, and how these are correlated to bee forage preferences and the land use characteristics in their foraging territory.

2. MATERIALS AND METHODS

2.1. Study areas

We used 23 commercial apiary sites located in southeastern Estonia in two consecutive years, 2013 and 2014. The number of apiaries

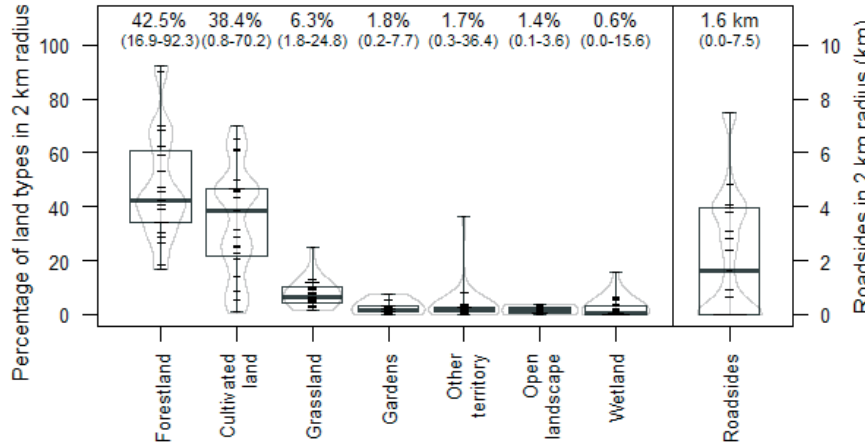

Figure 1. Distribution of studied land use parameters in 2 km radius. Box plots indicating the medians (strong horizontal lines), lower and upper quartiles, and minimum and maximum values, as well as violin plots indicating the empirical distributions, are presented. Short horizontal lines denote single apiaries. Medians are presented numerically, with minimum and maximum values in brackets.

differed between years (2013: N = 14; 2014: N = 19); some (N = 11) were used in both years. Apiaries were selected to represent variable land use types from almost 100% forest areas to 70% cultivated land. Land use types were calculated based on interactive maps of the Estonian Land Board using Arc-GIS (version 10.1, Esri, Redlands, CA, USA). The resulting GIS data was used to calculate area and proportion of each habitat type. In the calculations, both 2 or 4 km radiuses from each hive were taken into account. None of the main foraging ranges overlapped with any neighbouring apiaries studied.

Different land use types were represented over study areas. As in the whole of Estonia, forest covered the largest territories around the apiaries (Figure 1). The second largest land use type was cultivated land, varying in selected locations from nearly 0% to approximately 70%. There was a strong negative correlation between proportion of cultivated land and proportion of forest ($r = -0.87$; $p < 0.001$). The median coverage of all other measured land use types (grasslands, gardens, wetlands, and other smaller land use types) was 15.7%.

2.2. Sample collection

In total, 140 samples were collected. From each apiary, one queen-right and healthy (i.e. no disease symptoms) hive was selected. From the hive, samples of different materials (brood, nurse bees, honey, bee bread and corbicular pollen) were taken either once or twice per season according to flowering of winter (25 May – 2 June) or spring (4 – 15 July) oilseed rape. All samples were immediately placed in a cooler and transported to the laboratory where they were kept at -20 °C (except honey, which was kept at 5 °C) until analysis. The honey, brood and beebread samples were taken with pieces of relevant combs. Honey was extracted from comb via pressing it through sieves. Bee brood contained both larger larvae and pupae which were taken out from comb cells with forceps. From beebread samples, the empty parts of comb cells were cut off, though samples still contained wax, a factor which may have affected results, since wax is able to absorb various ACs (Lodesani et al., 2008; Orantes-Bermejo et al., 2010; Medici et al., 2015). To avoid in-hive contamination, in 2014 we collected only corbicular pollen gathered by honey bees during three-day course at flowering time of either winter or spring oilseed rape, using pollen traps at the entrance of the hive. Sampling was performed by researchers of the Estonian University of life Sciences whom received standardised training, to guarantee the similar handling of the samples.

2.3. Pesticide selection

Only pesticides commonly used in Estonia were selected to the study. The pesticide selection (N = 47 active ingredients, including those used for seed dressing only) was based on the Tartu County Farmers Association's pesticide ordering list for the years 2013 – 2014. Among the tested pesticides, 21 were herbicides, 15 fungicides, 10 insecticides and one plant growth regulator and retardant. The active ingredients analysed were: 2,4-D, alpha-cypermethrin, amidosulphuron, aminopyralid, azoxystrobin, clopyralid, cypermethrin, cyproconazole, deltamethrin, dicamba, dimethachlor, dimethoate, ethyl trinexapac, fenoxaprop-p-ethyl, fenpropidin, florasulam, fludioxonil, fluoxastrobin, flutriafol, fuberidazole, glyphosate, imazalil, imidacloprid, indoxacarb, iodosulfuron-methyl-sodium, lambda-cyhalothrin, MCPA, mefenpyr-diethyl, pencycuron, pinoxaden, prochloraz, propaquizafop, propiconazole, propoxycarbazone-sodium, prothioconazole, pymetrozine, pyroxsulam, quizalofop-p-

ethyl, spiroxamine, sulfosulfuron, tau-fluvalinate, tebuconazole, thiacloprid, triadimenol, triasulfuron and tribenuron-methyl.

2.4. Pesticide residue analyses

Pesticide residues in all beehive matrices were analysed by the Institute of Food Safety, Animal Health and Environment (BIOR). The method used for most of the compounds was the QuEChERS extraction method followed by quantification using GC-MS and UHPLC-MS/MS. Determination of glyphosate, aminopyralid and clopyralid were performed as single residue analyses by UHPLC-MS/MS. Details about applied standards of pesticides and materials used, sample preparations and exact methods, as well as performance of the methods, are described previously by Karise et al. (2017).

2.5. Determination of the botanical origin of pollen and honey

Botanical origin of the honey bee-collected pollen was determined from corbicular pollen collected by traps at the entrance of the hive, and from honey, both samples only from 2014. Pollen and honey samples were processed according to the standard acetolysis method (Moore and Webb, 1978). Pollen counts were based on a minimum of 300 grains. Pollen identification was made using a compound light microscope (400 × magnification). Pollen grains were identified by comparison to the reference pollen collection prepared at the Institute of Ecology at Tallinn University, and by the use of relevant literature (Moore and Webb, 1978; Reille, 1992; Moore et al., 1999).

2.6. Statistical analysis

The pesticide residue data by matrix type are presented as percent of samples contaminated, as well as median and maximum concentrations found. Using statistical software StatSoft (ver. 12, Inc. USA), we performed the chi-square test to compare the proportions of pesticides most commonly used in the region (21 herbicides, 15 fungicides, 10 insecticides) with those that were detected. The Kruskal-Wallis H test was used to assess the effects of sampling year and month on the number of different compounds, as well as on the total sum of residues detected in different matrices. The amount of pesticide residues in different matrices was tested using the parametric Wald chi-square test. Proportions of oilseed rape in pollen samples were compared using ANOVA.

The R 3.5.1 software (R Core Team, 2019) was used for Spearman correlation analysis assessing the strength of associations between a) cultivated land and forest, , b) total sum of pesticide residues and individual sums of insecticide, fungicide and herbicide residues, c) the proportion of different plant taxa and land use types in bee foraging area, d) proportion of different plant taxa and pesticide residues in pollen collected either in May or July, as well as from honey in July, and f) pesticide residues and proportions of land use types within 2 km radius around each hive. All test results were considered significant when $p < 0.05$.

3. RESULTS

3.1. Residues found

We found residues of 17 different active compounds from all three basic pesticide classes. The number of active ingredients detected did not reflect that which we searched for ($\chi^2(2) = 81.96$, df = 2, $p < 0.001$). Out of 21 herbicides, only 5 were detected; and of 15 fungicides, 4 were detected; but from 10 insecticides screened, 8 were detected (Figure 2). The compounds found in samples varied largely. From a total of 140 samples, tau-fluvalinate was the most frequently detected (39 samples), followed by thiacloprid (36), tebuconazole (22) and dimethoate (19). Low detection frequency of, or failure to detect, a particular compound may not reflect its presence in the bees' foraging environment, but is likely rather due to the material sampled or to the biochemical and physiochemical properties of different molecules.

3.1.1. Residues in different matrixes

The amount of pesticide residues in different matrices varied significantly ($\chi^2(4) = 9.67$, $p = 0.046$). Almost all beebread (95.2%) and pollen (96.6%) samples were contaminated, containing 1 – 8 and 1 – 6 different ACs per sample, respectively. Honey samples (69.6% contaminated) contained 1 – 3 compounds, brood (43.6%) 1 or 2 compounds and nurse bees (61.0%) only a single ACper sample. Most of the pesticide contents in beebread and pollen samples were due to insecticides and fungicides (Table 1), whereas in honey and nurse bee samples it was due to herbicides (Figure 2). The residues found in brood were usually at very low concentrations, and the significant correlation between the

Table 1. The proportion of samples with detected compounds (%), as well as median concentration (over samples exceeding the limit of detection, mg kg^{-1}) and maximum concentration (over all samples, mg kg^{-1}).

Compound	Log-Kow	Brood			Nurse bees			Honey			Beebread			Pollen		
		% (%>LOD)*	Median	Max	% (%>LOD)	Median	Max	% (%>LOD)	Median	Max	% (%>LOD)	Median	Max	% (%>LOD)	Median	Max
α-cypermethrin (I)	6.60	0% (0%)	-	0	0% (0%)	-	0	0% (0%)	-	0	19.0% (19.0%)	0.091	0.398	41.4% (41.4%)	0.070	0.214
Clopyralid (H)	1.06	0% (0%)	-	0	0% (0%)	-	0	27.3% (24.2%)	0.030	0.27	0% (0%)	-	0	3.4% (3.4%)	0.056	0.056
Cypermethrin (I)	6.60	0% (0%)	-	0	0% (0%)	-	0	0% (0%)	-	0	9.5% (9.5%)	0.259	0.398	41.4% (37.9%)	0.019	0.128
Deltamethrin (I)	6.20	0% (0%)	-	0	0% (0%)	-	0	0% (0%)	-	0	42.9% (28.6%)	0.013	0.016	0% (0%)	-	0
Dimethoate (I)	0.78	2.6% (0.0%)	-	0.002	0% (0%)	-	0	27.3% (0.0%)	-	0.005	14.3% (0.0%)	-	0	20.7% (6.9%)	0.034	0.042
Fludioxonil (F)	4.12	0% (0%)	-	0	0% (0%)	-	0	0% (0%)	-	0	4.8% (0.0%)	-	0	0% (0%)	-	0
Glyphosate (H)	-3.40	7.7% (0.0%)	-	0.034	44.4% (38.9%)	0.289	0.400	12.1% (6.1%)	0.059	0.062	0% (0%)	-	0	0% (0%)	-	0
λ-cyhalothrin (I)	7.00	0% (0%)	-	0	5.6% (5.6%)	0.037	0.037	0% (0%)	-	0	4.8% (0.0%)	-	0	17.2% (17.2%)	0.021	0.077
MCPA (H)	3.25	0% (0%)	-	0	5.6% (5.6%)	0.317	0.317	0% (0%)	-	0	4.8% (0.0%)	-	0	31.0% (6.9%)	0.015	0.019
Prothioconazole (F)	4.05	0% (0%)	-	0	0% (0%)	-	0	0% (0%)	-	0	9.5% (9.5%)	0.076	0.138	6.9% (6.9%)	0.285	0.546
Pymetrozine (I)	-0.18	0% (0%)	-	0	0% (0%)	-	0	0% (0%)	-	0	42.9% (0.0%)	-	0	0% (0%)	-	0

τ-fluvalinate (I, A)	7.02	33.3% (10.3%)	0.014	0.015	5.6% (0.0%)	-	0.007	15.2% (0.0%)	-	0.008	76.2% (52.4%)	0.014	0.036	13.8% (3.4%)	0.014	0.014
Tebuconazole (F)	3.70	5.1% (2.6%)	0.412	0.412	0% (0%)	-	0	9.1% (0.0%)	-	0.005	28.6% (9.5%)	0.039	0.062	37.9% (20.7%)	0.050	0.231
Thiacloprid (I)	1.26	0% (0%)	-	0	0% (0%)	-	0	18.2% (6.1%)	0.014	0.014	42.9% (9.5%)	0.014	0.017	72.4% (44.8%)	0.029	0.236
2,4-D (H)	2.81	0% (0%)	-	0	0% (0%)	-	0	3.0% (0.0%)	-	0.002	0% (0%)	-	0	13.8% (3.4%)	0.041	0.041
Azoxystrobin (F)	2.50	0% (0%)	-	0	0% (0%)	-	0	3.0% (3.0%)	0.031	0.031	0% (0%)	-	0	3.4% (3.4%)	0.040	0.040
Dicamba (H)	2.21	0% (0%)	-	0	0% (0%)	-	0	0% (0%)	-	0	0% (0%)	-	0	3.4% (3.4%)	0.020	0.020

* % - the proportion of samples with detected compounds; $\%_{>LOD}$ - the proportion of samples with detected compounds exceeding the limit of detection 0.01 mg kg^{-1} (except 0.05 mg kg^{-1} for glyphosate); LogKow - logarithm of Octanol-Water distribution coefficient; I – insecticide; H – herbicide; F – fungicide.

total amount of pesticides and the total amount of fungicides is likely due to a high concentration of tebuconazole detected in one sample, and therefore cannot be considered reliable.

We understand that tau-fluvalinate residues found in beebread may partly originate from acaricides used as veterinary treatments in the hive. Being a fat-soluble compound, tau-fluvalinate persists in wax, and we could not prevent the occurrence of bits of comb wax in beebread.

3.1.2. Effect of year and collection time

We found no variation in the number of compounds per sample in brood or honey. The total sum of residues did not differ in the brood, but was significantly higher in honey in 2013 compared to 2014. Comparison of beebread (2013) and pollen (2014) samples revealed the opposite result: the sum of residues was higher in 2014 (Table 2). When looking only apiaries, that were analysed both years, the effect of year persisted: Brood: $H (1, N = 12) = 2.435241$ $p = 0.1186$; Honey: $H (1, N = 20) = 4.192537$ $p = 0.0406$; Pollen: $H (1, N = 30) = 3.985113$ $p = 0.0459$.

During the flowering of winter oilseed rape, the pollen samples contained 3 – 8 pesticides in 2013 and 1 – 6 in 2014, whereas during the flowering period of spring oilseed rape, the number stayed between 0 – 4 in both years. The sum of all residues was significantly higher in May in 2013, but not in 2014.

3.1.3. Effect of the hive

When looking at different matrices from the same hive, we saw only a few repetitions of the same ACs. Tau-fluvalinate was found mostly in beebread, but not so often in other matrices. There were some pairs of the nurse bee and brood samples containing glyphosate residues (about ten times lower concentration in brood than in nurse bees). However, these results did not match with the glyphosate findings from honey.

3.2. Pollen origin

Plant species determination (data from 2014) from the pollen collected by honey bees revealed that there was a noticeable variability among all gathered pollen species. Oilseed rape pollen comprised 51.9% of total,

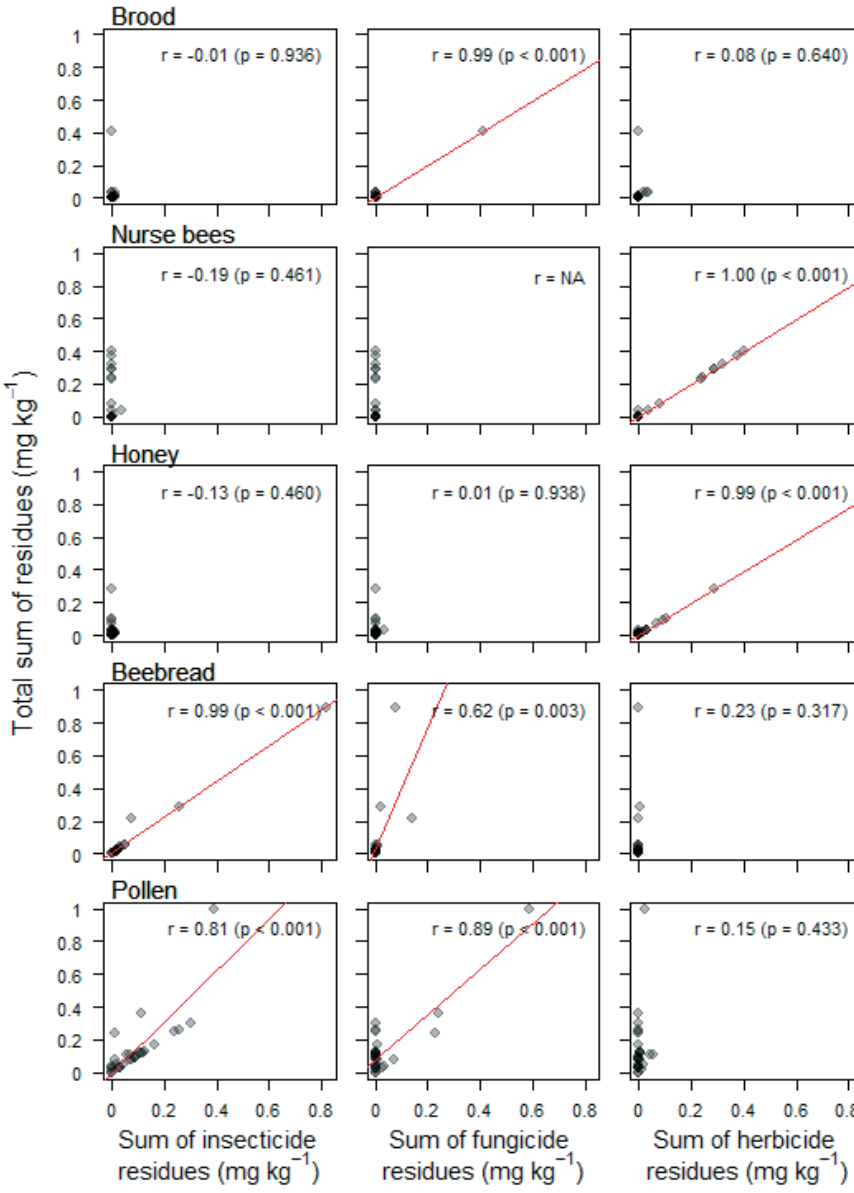

Figure 2. Relationships between total sum of residues and sum of insecticide, fungicide and herbicide residues by matrixes. Linear correlation coefficients (with p-values) are presented numerically. Statistically significant relationships ($p < 0.05$) are marked with the linear regression line.

Table 2. The Kruskal-Wallis H statistic (KW-H) and p-values for the effects of sampling year or collection time on numbers of different ACs detected and the cumulative amount of residues in repeatedly collected matrices.

Effect	Matrix	Number of different compounds		The total sum of residues	
		KW-H	P-value	KW-H	P-value
Sampling year	Brood	0.86	0.35	2.68	0.10
	Honey	0.61	0.44	4.91	0.03
	Beebread/pollen	0.11	0.74	9.47	0.002
Sampling month	Brood	n.a.*	n.a.	0.03	0.86
	Beebread	9.24	0.002	8.48	0.004
	Pollen	2.58	0.11	0.21	0.65

* n.a. – not available

followed by other plant taxa as shown in figure 3 (A and B). Interestingly, there were no significant correlations between the representation of any plant taxa in collected samples and land use types. The presence of oilseed rape pollen in samples did not correlate with the percentage of any land use type within either a 2 km or 4 km radius. Despite the absence of positive statistical correlation, the species was clearly preferred, especially in July [medians (min, max) May: 35.3 (0.0, 83.5), July: 82.2 (28.2, 88.7)]. Nevertheless, in May, there were some correlations between the presence of some other plant taxa and land use type, as shown in figures 3A and 3B.

Honey samples obviously reflect a much longer time period compared to pollen samples, and thus a greater diversity of plant taxa can be detected in honey samples (Figure 3C). It is noteworthy, that Estonian beekeepers harvest honey mainly once a year (in august) and thus the honey harvested originates from various plant taxa blooming in the foraging season. As expected, the most abundant species of pollen found in our honey samples within both 2 km and 4 km radiuses belonged also to oilseed rape (25.9%), followed by plants from Asteraceae family (Figure 3C).

3.3. Correlations between the percentage of plant taxa and pesticide residues in pollen samples

The composition of ACs found in May was different from that of July: in 2014, at the end of May, 13 active ingredients were recognised; at the beginning of July, 8 active ingredients were recognized. Most of the ACs

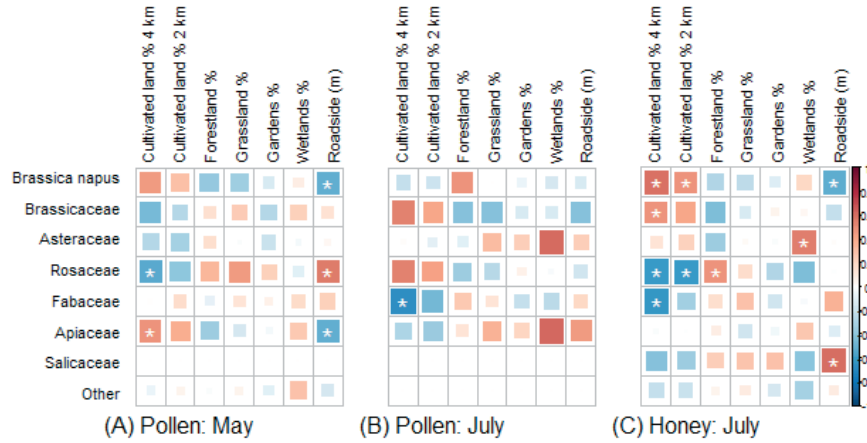

Figure 3. Correlations between the presence of plant taxa in hive matrices (A = pollen, May; B = pollen, July; C = honey, July) and land use types in May and July 2014. Red colours indicate positive and blue colours negative correlation; the darker the colour, the stronger the correlation; asterisks indicate statistically significant correlations ($p < 0.05$). Pollen samples reflect a 3-day time period either during flowering of winter oilseed rape (May) or spring oilseed rape (July), whereas honey samples reflect the whole foraging season from April to July.

were not correlated with the percentage of any particular plant taxa found in pollen. In May, we saw a significant negative correlation between percentage of oilseed rape pollen and the amount of two pyrethroids, alpha-cypermethrin and cypermethrin, whereas we saw a significant positive correlation between percentage of oilseed rape pollen and residues of the organophosphate insecticide dimethoate (Figure 4A). Dimethoate residues were positively correlated with pollen from Family Apiaceae, too. Residues of the neonicotinoid insecticide thiacloprid were also positively correlated with pollen from plants belonging to Apiaceae. Total amount of herbicides was positively correlated with pollen of Fabaceae. In May, the total sum of ACs showed a significant negative correlation with winter oilseed rape. In July, we found only one significant correlation between different plant taxa and an active ingredient: the amount of pollen from Apiaceae in honey bee forage was positively correlated with thiacloprid residues (Figure 4B). Positive but not significant correlations occurred also between: oilseed rape pollen and alpha-cypermethrin; cypermethrin and MCPA; Fabaceae pollen and prothioconazole; tau-fluvalinate and thiacloprid; and Asteraceae pollen and lambda-cyhalothrin.

Based on honey samples, we found significant positive correlations betweenFabaceae and thiacloprid, as well as between Apiaceae and tau-fluvalinate (Figure 4C), while a significant negative correlation was seen between Asteraceae and tebuconazole. The sum of herbicides was positively correlated with non-crop Brassicaceae.

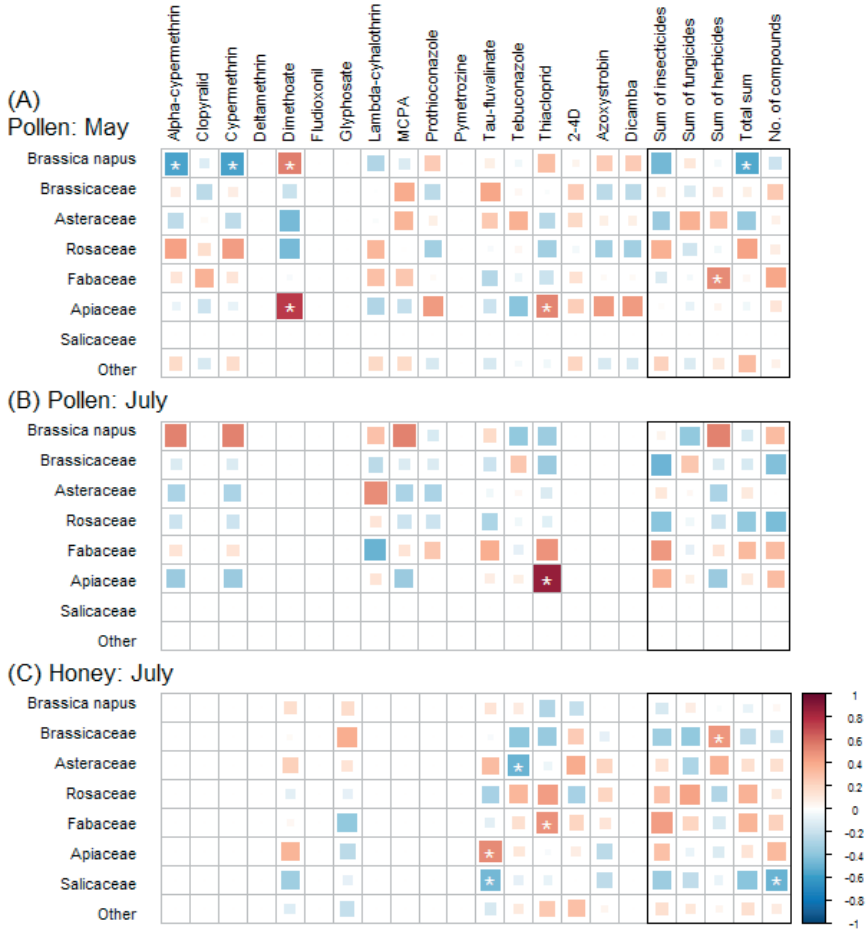

Figure 4. Correlations between the presence of plant taxa in hive matrices (A = pollen, May; B = pollen, July; C = honey, July) and land use types in May and July 2014. Red colours indicate positive and blue colours negative correlation; the darker the colour, the stronger the correlation; asterisks indicate statistically significant correlations ($p < 0.05$). Pollen samples reflect a 3-day time period either during flowering of winter oilseed rape (May) or spring oilseed rape (July), whereas honey samples reflect the whole foraging season from April to July.

3.4. The effect of habitat factors on pesticide residues

There were no significant correlations between pesticide residues detected in samples and different land use types (all correlations $r < 0.15$ and $p > 0.05$). A similar result was obtained when calculating separately for honey, beebread or pollen.

4. DISCUSSION

Pesticidal active compounds found in different beehive matrices varied within collection month and among years. Oilseed rape is grown using intensive agricultural techniques, demanding active management of pests and pathogens. However, despite the fact that a large amount of pollen at both collection times originated from oilseed rape, we observed no positive correlation between amounts of pesticide residues and the proportion of the cultivated land around the hives.

4.1. Pesticide residue distribution in time and across matrices

The need for pest control is variable by geographic region, crop, month and year (Böhme et al., 2018). Furthermore, the same pesticides should not be used in consecutive years, in order to avoid the development of resistance in pest populations (US EPA, 2017). These variable practices lead to variable findings of pesticide residues, as has been demonstrated in honey (Karise et al., 2017), pollen and beebread (Beyer et al., 2018; Tong et al., 2018).

Honey bees have been used as the bio-indicators of heavy metal pollution in the environment (Celli and Maccagnani, 2003; Zhelyazkova, 2012; Skorbiłowicz et al., 2018). However, to obtain comparable results between studies, clever sample collection design is needed (Herrero-Latorre et al., 2017). Although massive die-outs of honey bees may indicate misuse of insecticides, it is not easy to locate the source field of the applied pesticides (Henry et al., 2012; Estonian Agricultural Board, 2015). This may be due to the variable ability of ACs to absorb into different materials. The results of the present study reproduce the variability of pesticide residues in bee products within and among years, and between matrices. We found that pollen and beebread samples are primarily contaminated with insecticides, and to a lesser extent of fungicides. Madej et al. (2018) have stated that most pesticides are lipophilic,

accumulating better in fatty materials. Indeed, we found that primarily lipophilic pesticides (pKow > 3.7) occurred in beebread and pollen, both containing greater than 10% fat. Fat content of honey is relatively low, and it contained mostly residues of herbicides, a result similar to that obtained in an Australian study (Manning, 2018). Some hydrophilic pesticides (e.g. glufosinate, glyphosate, maleic hydrazide, chlormequat, diquat, mepiquat) can also become lost during general sample processing for multi-residue analyses, and therefore require single residue analyses (Kaczyński, 2017). Because of difficulties in determining the most common herbicides contaminating samples (Kaczyński, 2017; Thompson et al., 2019), insecticide and fungicide residues are more often considered in multi-residue analyses of honey (Panseri et al., 2014; Rodríguez López et al., 2014; Al Naggar et al., 2015; Christodoulou et al., 2015; Chiesa et al., 2016; Juan-Borrás et al., 2016; Souza Tette et al., 2016). The bias of chemical analyses towards insecticides and fungicides has created an opinion about the presence of relatively few pesticide residues in honey. However, in a review by Thompson et al. (2019), when the glyphosate was intentionally searched for, contamination in honey occurred in 9 – 98% of samples.

We saw that sample collection year significantly affected the total sum of residues in honey and pollen, but not in brood. This indicates that brood may be well protected from these toxic compounds. Most but not all of the detected residues occurred at very low concentrations, staying close to the limit of detection. All the studied beehive matrices were contaminated with a mixture of ACs, and sometimes at concentrations that may potentially affect either behaviour or physical health of the individuals (Muljar et al., 2012; Raimets et al., 2017). Herbicide residues found in nurse bees or honey probably do not cause mortality, but can affect honey bee gut microbiota (Motta et al., 2018).

In this study, insecticides were detected more than fungicides in both pollen and beebread samples. Such a difference may be the result of differences in pesticide application due to climatic variability. Agricultural crops are more susceptible to fungal pathogens in warmer climates. Indeed, in Florida and California, fungicide residues accounted for most of the pesticide content in pollen samples (Mullin et al., 2010). Still, results vary between studies, and relationships with climatic conditions are unclear. For instance, pollen data from Luxemburg (Beyer et al., 2018) showed contamination by insecticides, fungicides and herbicides,

while during the same year, a study from Spain (Calatayud-Vernich et al., 2018) reported only acaricides and insecticides, in honey bee collected pollen. Similar to our results, insecticides were detected more than fungicides and herbicides in pollen samples in a Chinese study (Tong et al., 2018). To gain more insight into the distribution of pesticides by region, a longer sampling interval within and among years, standardised sample collection procedures, and chemical analyses of bee products over large areas are needed.

Our brood samples were relatively residue-free, which may be explained by nurse bees acting as filters for brood (DeGrandi-Hoffman et al., 2013). Still, during later life stages, honey bee larvae also feed directly on beebread. There are opposing results regarding whether honey bee larvae possess higher (du Rand et al., 2017) or lower (Fine and Mullin, 2017) detoxification abilities. Although nurse bees consume most of the pollen brought into the hive to produce royal jelly for larvae and the queen, the level of contamination with insecticides and fungicides was very low in nurse bees. We found only one AC in nurse bees – glyphosate, present in 44% of samples. Glyphosate is the most widely used AC globally, and even a single treatment applied to a crop may result in detectable residues in both nectar and larvae (Thompson et al., 2014). Honey bees were observed to prefer glyphosate-contaminated sugar syrup, possibly accounting for the frequency with which this pesticide occurs as a hive contaminant (Liao et al., 2017). Although glyphosate travels through bees and honey into the larvae, we did not see this compound in honey, nurse bees or larvae from the same hives.

4.2. The botanical origin and landscape

Honey bee foraging distances are variable in size, depending on the availability of profitable forage areas (Beekman and Ratnieks, 2000) as well as the colony's nutritional requirements (Danner et al., 2017). To provide a colony with sufficient food, superabundant floral resources are preferred. In Estonia, one important food source for honey bees is oilseed rape, which represents an important source for both pollen (Danner et al., 2017) and nectar (Puusepp and Koff, 2014). We saw during midsummer that honey bees foraged primarily on oilseed rape, at least for pollen, whereas in May their diet was broader. This indicates that during the short flowering period of spring oilseed rape, other abundant floral resources may be scarce.

Pesticide residue content in honey, however, suggests that the proportion of oilseed rape pollen grains in honey and cultivated area are positively correlated. Oilseed crops represented 8 – 17% of all cultivated crops within the study areas (Statistics Estonia, 2019). We suspect that in locations where the cultivated area was 10 – 20% of the whole territory (a circle with 4 km radius), the distance to the nearest oilseed rape field was even greater. This also indicates that the 3 km buffer zone (EU Commission Regulation, 2008) around an organic apiary does not guarantee that honey bees do not forage on conventionally grown crops.

Other bee-attractive crops grown in Estonia are either not flowering at the same time as oilseed rape, or cannot compete with oilseed rape as a source of pollen and nectar. Legume crops covered 6 – 12% of cultivated area in both 2013 and 2014, and are negatively correlated with the proportion of oilseed rape, in the study region.

4.3. Origin of residues and relationship with land use type

In earlier years, the most important oilseed rape pest, the pollen beetle *Brassicogethes aeneus* Fabricus (Veromann et al., 2006), had caused considerable damage only to spring and not winter oilseed rape. The situation has changed in that the *B. aeneus* emerges earlier (Junk et al., 2016), and some additional pests (e.g. weevils of *Ceutorhynchus sp.*) are more abundant. Consequently, farmers spray winter oilseed rape as well (farmers' observations). Our results show higher AC loads in honey bee forage during the flowering of winter oilseed rape, compared to spring oilseed rape. However, looking at the complex of pesticidal active ingredients, we did not see any relationship with oilseed crops in particular. This may suggest that different ACs are used at a different times of the season to control a broad range of pests.

Most of these ACs are used on many crops including cereals, legumes and crucifers (Estonian Agricultural Board, 2019a). The pyrethroid insecticide lambda-cyhalothrin is commonly used on several crops including clovers, peas and beans. Although both alpha-cypermethrin and cypermethrin tended to be positively correlated with the amount of oilseed rape in honey bee forage, there is still no clear connection, as farmers use these compounds more often against aphids. Pyrethroids are losing their efficacy against *B. aeneus*, and are being replaced by systemic compounds like pymetrozine or thiacloprid. Thiacloprid is commonly used against oilseed rape pests, but also against insect pests in other agroecosystems in-

cluding wheat, orchards and cotton. According to our results, thiacloprid was positively correlated with Apiaceae pollen both in May and July, likely because of wildflowers (e.g. ground elder, *Aegopodium podagraria* L.) that may be common on field edges or inside the cropped fields. Farmers are allowed to spray thiacloprid on flowering crops or weeds during the time of day when bees are not active. However, this prescription does not prevent damage to bee populations, as thiacloprid is systemic and stays in plant tissues for more than seven days (Estonian Agricultural Board, 2019a). Pymetrozine was the only detected AC clearly associated with oilseed rape, but the concentrations found were too low to create a statistical model. This may be because the use of pymetrozine is restricted to the period of formation of flower buds, and this compound causes immediate and irreversible cessation of pest-feeding (Fuog et al., 1998).

The organophosphate insecticide dimethoate is highly toxic to bees, and therefore not allowed for application to flowering crops in Estonia. However, misuse occurs sometimes, resulting in massive die-outs of honey bees, as has been experienced several times in Estonia (Estonian Agricultural Board, 2019b). In this study, no misuse was indicated, and nearly all colonies survived the winters. Since the formulations containing dimethoate are commonly used on cereals during the same time period, contamination of bee-collected pollen likely originated in wildflowers located in field margins, or from pesticide drift reaching non-target areas. We found that the amount of dimethoate was positively correlated with the proportion of Apiaceae and oilseed rape in pollen sampled in May.

All the detected fungicides are used on several crops including oilseeds and legumes, which also are important for pollinators. The herbicide clopyralid may be tightly connected to oilseed rape, since this AC is commonly used on oilseeds from the early growth stage to the pre-flowering period against broad-leaved weeds, but is commonly used on other crops as well (Estonian Agricultural Board, 2019a). Another herbicide, glyphosate, is the most regularly sold and widely used AC in Estonia, with a wide range of use: on fields with minimal or zero tillage systems, before tillage of a new field, but also to control wildflower growth on roadsides and railway embankments (Steinmann et al., 2012). In addition, this is a common pesticide used in private gardens against excessive grass growth. The other detected herbicides are used on peas, clovers (MCPA) and cereals (dicamba and 2,4-D) (Estonian Agricultural Board, 2019a), and may impact pollinators via affecting on adjacent plant communities.

5. CONCLUSION

Using beehive matrices to estimate environmental contamination levels requires highly sophisticated sampling methods due to several aspects of honey bee biology (e.g. variable pesticide inflow, dynamic nutritional requirements of honey bee colonies, specific physico-chemical characteristics of different ACs absorbing into different beehive materials).

Oilseed rape is a preferred source of both pollen and nectar for honey bees. This crop commonly offers a superabundant food supply, at least in July during the time of most honey inflow. However, the ACs detected in this study are related to a wide range of plants, including both crops and non-crop sources. Despite the high abundance of oilseed rape in honey bee-collected pollen, oilseed rape pollen was not directly correlated to any land use type or pesticidal active ingredient found in beehives.

Author contribution

RR, RK and MM did the experiment design and manuscript writing. HV, PP, IK and RR worked through the agricultural databases and carried through the interviews with farmers on their habitats of pesticide usage. HV, PP, IK and RR also selected the study sites and managed the honey bee hives and collected the data. TK, RK and MM designed and performed the statistical analyses and wrote the relevant text. VB and IP designed the chemical methods and carried through the chemical analyses and calculations and also wrote the relevant text. AB and LP made pollen analyses and contributed to the text writing.

Conflict of interest

The authors report no conflicts of interest.

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II

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ARE PESTICIDE RESIDUES IN HONEY RELATED TO OIL-SEED RAPE TREATMENTS?

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Are pesticide residues in honey related to oilseed rape treatments?

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HIGHLIGHTS

- The amount of pesticide residues in honey can vary largely between the years.
- The residues in honey tend to be connected to those used in oilseed rape fields.
- Clopyralid and glyphosate residues prevailed in honey samples.
- The concentrations found do not pose any health risk to consumers.
- The concentrations probably do not cause any acute toxicity to honey bees.

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ABSTRACT

Pesticide treatments before and during the flowering of honey bee forage crops may lead to residues in honey. In northern regions oilseed rape belongs to the main forage crops that is mostly cultivated by means of intensive agriculture, including several pesticide treatments. However, in addition to the focal forage crops, pesticides from non-forage crops can spread to wild flowers around fields, and thus the residues in honey would reflect the whole range of pesticides used in the agricultural landscape. The aim of our study was to clarify which currently used pesticides are present in honey gathered from heterogeneous agricultural landscapes after the end of flowering of oilseed crops.

Honey samples (N = 33) were collected from beehives of Estonia during 2013 and 2014, and analysed for residues of 47 currently used agricultural pesticides using the multiresidue method with HPLC-MS/MS and GC-MS and a single residue method for glyphosate, aminopyralid and clopyralid. Residues of eight different active ingredients with representatives from all three basic pesticide classes were determined. Although no correlation was detected between the cumulative amount of pesticide residues and percent of oilseed crops in the foraging territory, most of the residues are those allowed for oilseed rape treatments. Among all pesticides, herbicide residues prevailed in 2013 but not in 2014. Despite the relatively small agricultural impact of Estonia, the detected levels of pesticide residues sometimes exceeded maximum residue level; however, these concentrations do not pose a health risk to consumers, also acute toxicity to honey bees would be very unlikely.

1. Introduction

Using honey as natural food sets high demands on its quality. However, honey production occurs hand-in-hand with agricultural activities, and pesticide residues have been detected in honeys from several countries at varying levels, sometimes even exceeding the maximum residue levels (MRL) allowed (Souza Tette et al., 2016).

Pesticides can enter beehives via several routes. Hive treatments using medical products to combat honey bee parasites and

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pathogens bring about residues in wax and other bee products (Kujawski and Namieśnik, 2011; Nakajima et al., 2015). Honey bees collect pollen and nectar from treated crops: they might not avoid freshly treated fields even if the product used has been labelled as being repellent to bees (Karise et al., 2007). Foraging outside the fields may also result in contaminated food resource through spray drift from fields to wild vegetation (Long and Krupke, 2016). It has been convincingly demonstrated that pesticides used on fields can drift a long way to neighbouring areas (Krupke et al., 2012; Hladik et al., 2016; Long and Krupke, 2016), thus contaminating the pollen and nectar of wild flowers, which in turn may lead to contaminated honey production even in organic apiaries, as described in Italy (Chiesa et al., 2016). In addition to currently used pesticides, field soil tends to retain many chemicals used throughout (Kumar et al., 2016; Lozowicka et al., 2016; Zhang et al., 2016), and moreover, traces may occur in every plant product including nectar and pollen (Malhat et al., 2015; Chiesa et al., 2016).

Analyses of pesticide residues in honey have been carried out in several countries (reviewed by Souza Tette et al., 2016). An important set of studies analysed honey for contamination by organochlorines, many of which are banned (Panseri et al., 2014; Al Naggar et al., 2015; Chiesa et al., 2016). However, although this data is interesting, it does not lead to any understanding of the consequences of those pesticides used nowadays when pyrethroids and neonicotinoids are becoming more and more popular. We know of no up-to-date survey on honey contamination by a broader spectrum of currently used pesticides. For Nordic areas, there is only one study which analyses honey and this considers the presence of four pesticide residues of neonicotinoid insecticides (Laaniste et al., 2016), where it is shown that the frequency of pesticide residues in honey was correlated with the year-wise increase in product importation.

As in other Nordic countries, the pesticide input into Estonian agriculture is relatively low, being less than 1 kg ha^{-1} of utilised agricultural area (Eurostat, 2015), whereas in most Central European countries like France, Germany, Belgium and the Netherlands, the amount of pesticides sold is over 2 kg ha^{-1}. In Estonia, more than half of the country's territory is covered with forests and other wooded lands (Eurostat, 2015). This, and the low pesticide input, makes people assume that the nectar from wild and presumably unpolluted flowers should dilute the nectar from cultivated plants to a level where residues are no longer detectable.

Estonian honey is polyfloral; however, Brassicaceae pollen belongs to the four most common plant species found from honey samples (Puusepp and Koff, 2014). Most of the Brassicaceae pollen probably belongs to cultivated oilseed crops, from which most are grown by means of conventional agricultural methods. The pesticide treatment suggested for oilseed rape starts with soil preparation using herbicides, followed by sowing dressed seed to protect the seedlings against fungal diseases and flea beetles. Later, several treatments against other insect pests and phythopathogens are suggested. As pre-harvest treatment, glyphosate is suggested to reduce harvesting losses. Due to the large content of Brassicacea pollen in Estonian honey (Puusepp and Koff, 2014), we hypothesize that the possible residues found in honey reflect those used in oilseed rape agrotechnology. However, there is evidence that different groups of pesticides are correlated differently with forage crops in foraging ranges of honey bees (McArt et al., 2017). Therefore, we aimed to clarify which of the currently used 50 pesticides are present in honey gathered from heterogeneous agricultural landscapes after the flowering of oilseed crops.

2. Material and methods

2.1. Study location

Honey samples were gathered from Eastern and Southern Estonia (Ida-Viru, Tartu, Põlva and Valga Counties) in 2013 (N = 14) and 2014 (N = 19). This area is representative of typical agricultural landscapes in Estonia with mostly intensively managed fields, forested areas and human settlements. Among other field crops, both winter and spring oilseed rape are often grown in Estonia, and both belong to the common forage crops of honey bees. Within a 2 km radius of each hive there is on average 34.6 ± 20.7% cultivated land (min. 0.81%, max. 70.2%), 48.1 ± 20.6% forest, 5.3 ± 7.6% waste and vacant land, 7.6 ± 5.0% grassland and 2.1 ± 3.6% garden. The average oilseed crop coverage within the foraging territory remained between 0 and 12.9%.

2.2. Pesticide selection

The 47 active ingredients analysed were selected for the survey as being the most commonly used in Estonian fields according to the pesticide ordering lists of the Tartu County Farmers Association for the year 2013–2014. These include the most commonly used contemporary herbicides (21), fungicides (15) and insecticides (10), and plant growth regulator and retardant (1). The active ingredients searched for were: 2,4D, alpha-cypermethrin, amidosulphuron, aminopyralid, azoxystrobin, clopyralid, cypermethrin, cyproconazole, deltamethrin, dicamba, dimethachlor, dimethoate, ethyl trinexapac, fenoxaprop-p-ethyl, fenpropidin, florasulam, fludioxonil, fluoxastrobin, flutriafol, fuberidazole, glyphosate, imazalil, imidacloprid, indoxacarb, iodosulfuron-methyl-sodium, lambda-cyhalotrin, MCPA, mefenpyr-diethyl, pencycuron, picloram, pinoxaden, prochloraz, propaquizafop, propiconazole, propoxycarbazone-sodium, prothioconazole, pymetrozine, pyroxsulam, quizalofop-p-ethyl, spiroxamine, sulfosulfuron, tau-fluvalinate, tebuconazole, thiacloprid, triadimenol, triasulfuron and tribenuron-methyl.

2.3. Sample collection and handling

A total of 33 honey samples were collected from beehives in the eastern and southern part of Estonia (Tartu County and its near vicinity) during 2013 and 2014 for analysis of pesticide residues. Each honey sample originated from a different apiary, each of which consisted of 10–20 honey bee hives. The sampled hive was selected randomly for testing. The distance between sampled apiaries was at least 4 km in 2013 and at least 8 km in 2014 to preclude overlapping of the main forage area. The samples were gathered from honeycombs within beehives during the honey harvest in mid-July after the end of oilseed rape flowering. Due to the funding allocated for this study, it was decided to concentrate only on honey samples, and in order to cover more apiaries from the largest possible territory, we sampled only one hive per apiary. The honey was extracted from the comb wax and thereafter kept at 5 °C until analysis.

2.4. Chemicals and materials

The reference standards of pesticides were purchased from AccuStandard (New Haven, USA) and Dr. Ehrenstorfer (Germany). HPLC grade acetonitrile and methanol were purchased from Merck-Millipore (Darmstadt, Germany). ACS grade formic acid (≥96.0%), acetic acid (glacial, ≥99.85%), and ammonium formate (99%) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Ultrapure deionised water was generated by a Millipore Milli-Q™ system

(Billerica, MA, USA). A buffer-salt mixture (1 g trisodium citrate dihydrate, 1 g sodium chloride, 0.5 g disodium hydrogen citrate sesquihydrate and 4 g of anhydrous magnesium sulphate) and a mixture of dSPE (900 mg anhydrous magnesium sulphate, 150 mg PSA and 150 mg C18E) were obtained from Phenomenex (Torrance, CA, USA).

Stock solutions of approximately 1000 mg L^{-1} concentration were prepared by weighing 10 mg of standard in a 10 mL graduated flask and dissolving it in acetonitrile. The purity of the standard was taken into account in the preparation of standard solutions of final concentration. The mix of working standard solution with a concentration of 0.01 mg L^{-1} was prepared by diluting the appropriate volume of stock solution in acetonitrile. The stock and working standard solution were stored at −20 °C.

2.5. Sample preparation

Different sample extraction and detection procedures were used for analysis of the selected pesticides. Most compounds were analysed using QuEChERS extraction methodology followed by detection using GC-MS and UHPLC-MS/MS. Analysis of glyphosate, aminopyralid and clopyralid was performed as single analyses using extraction with methanol.

5.0 ± 0.1 g of the sample was weighed into a 50 mL polypropylene centrifuge tube. For calibration and quality control samples, the standard solutions were added at the appropriate spiking level. Deionised water (10 mL) and acetonitrile (10 mL) were both added and the tubes were shaken vigorously by hand for 1 min. Then a salt mixture of 4 g of magnesium sulphate, 1 g of sodium chloride, 1 g of trisodium citrate dihydrate, and 0.5 g of disodium hydrogen citrate sesquihydrate was added, the tubes were closed and immediately shaken by hand for 1 min and centrifuged for 5 min at 4500 rpm. An aliquot of 8 mL of supernatant was transferred into a 15 mL PP centrifuge tube and frozen out at −80 °C for 30 min using a Heto Ultra freeze (Thermo Fisher Scientific, USA), followed by centrifugation of the resulting organic sample fraction for 5 min at 4500 rpm. For pesticides with acidic groups that interact with amino sorbents such as PSA, an aliquot of 250 μL of the raw extract was mixed with 500 μL of the mobile phase A (5 mM ammonium formate and 0.1% formic acid in water) and analysed by UHPLC-MS/MS using negative electrospray ionisation mode. For further clean-up procedure, 6 mL of extract was transferred into 15 mL PP tubes containing 900 mg anhydrous magnesium sulphate, 150 mg PSA and 150 mg C18E. The tubes were shaken vigorously for 30 s and centrifuged for 5 min at 4500 rpm. For analysis with GC-MS, 5 mL of cleaned extract were evaporated in a water bath (40 °C) under a gentle nitrogen stream. The samples were reconstituted in 100 μL of acetonitrile and transferred into screw cap vials with inserts. For UHPLC-MS/MS analysis using ESI in positive ionisation mode, an aliquot of 250 μL of cleaned extract was mixed with 500 μL of the mobile phase A. When final sample extracts were misty, they were filtered through 0.22 μm PVDF centrifuge filters before transferring them into autosampler vials for analysis.

For analysis of glyphosate, aminopyralid and clopyralid, 5.0 ± 0.1 g of samples were weighed into a 50 mL polypropylene centrifuge tube, then 10 mL of water and 10 mL of methanol were added for extraction. The samples were shaken for 20 min and centrifuged for 10 min at 4500 rpm. An aliquot of extract was transferred to an autosampler vial for analysis by UHPLC-MS/MS.

2.6. GC-MS analysis

The sample extracts in acetonitrile were analysed on an Agilent HP 6890 gas chromatograph coupled with an HP 5973 mass spectrometer (Agilent Technologies, CA, USA) operating in SIM mode (Table 1). The capillary column used was Agilent DB-5MS (30 m × 0.25 mm × 0.25 μm). Operating conditions: the carrier gas was helium at a constant flow rate of 1.2 mL min^{-1}, injector temperature of 250 °C and the interface temperature was 250 °C. The initial oven temperature was 60 °C (held for 2 min), then increased to 150 °C at a rate of 30 °C min^{-1} (held for 2 min), then increased to 240 °C at a rate of 3 °C and held for 2 min, afterwards increased to 270 °C at a rate of 10 °C and held for 30 min. The total analysis time was 72 min. Injection volume was 1 μL.

2.7. UHPLC-MS/MS analysis

An Acquity UHPLC system (Waters, USA) coupled to QTrap 5500 (AB SCIEX, USA) equipped with an electrospray ionisation source was used for the analysis of pesticides in honey. The parameters of the ion source were as follows: source temperature was set at 500 °C, ion spray voltage 5.00 kV for positive ionisation mode and −4.50 kV for negative, curtain gas nebulizer 45 psi, ion source gas 1 (GS_1) 40 psi, and ion source gas 2 (GS_2) 60 psi. The analysis was performed by multiple reaction monitoring (MRM) in the positive and negative ionisation modes. Table 2 lists the analyte dependent parameters – MRM transitions, collision energies (CE) and declustering potential (DP). The control of the instrument conditions and the data processing were performed using Analyst 1.6 software (AB SCIEX, USA).

Chromatographic separation for most pesticides (except glyphosate, aminopyralid and clopyralid) was performed on a Kinetex C18 analytical column (50 × 3.0 mm, 1.7 μm) from Phenomenex. The mobile phase (A) consisting of 5 mM ammonium formiate and 0.1% formic in water and (B) acetonitrile was delivered at the flow rate of 0.4 mL min^{-1}. A gradient program was used: 20% of mobile phase (B) was used from 0 to 1.0 min, 20% (B) to 90% (B) from 1.0 to 10.0 min, maintained at 90% (B) for 1 min, then decreased back to 20% (B) at 11.0 min and finally the column was re-equilibrated with 20% (B) from 11.0 to 15.0 min. An aliquot of 10 μL of the extract was injected. The column and autosampler were maintained at 30 °C and 10 °C, respectively.

Aminopyralid and clopyralid were analysed on a Luna SCX analytical column (50 × 4.6 mm, 5 μm) from Phenomenex. The mobile phase (A) consisted of 5 mM ammonium formiate and (B) methanol was delivered at the flow rate of 0.6 mL min-1with isocartic elution mode (40% of A and 60% of B). The time of analysis was 5 min, the injection volume was 10 μL and the column and autosampler were maintained at 30 °C and 10 °C, respectively.

Glyphosate was analysed on a Hypercarb analytical column (100 × 2.1 mm, 5 μm) from Thermo Scientific (MA, USA). The mobile phase, consisting of 1% acetic acid in water, was delivered at the flow rate of 0.3 mL min^{-1}. The time of analysis was 10 min, the injection volume was 10 μL and the column and autosampler were maintained at 40 °C and 10 °C, respectively.

3. Results and discussion

3.1. Performance of the method

The performance of the method was evaluated according to the EC guidance document SANCO/12571/2013. The method showed good linearity with the determination coefficients, higher than 0.990 for all compounds included in the study. The mean variation of coefficients for repeatability of the method ranged from 3.0% to 16%, and the recovery ranged from 78% to 115%.

The limit of quantification (LOQ) for which the S/N ratio exceeds 10 was assumed at a concentration level of 0.010 mg kg^{-1} for all pesticides with the exception of aminopyralid, clopyralid,

Table 1
Acquisition parameters for the selected pesticides analysed by GC-MS.

Analyte	Ions selected for monitoring (*m*/*z*)	Retention time (min)
Cypermethrin I	163, 181, 165, 91	42,02
Cypermethrin II	163, 181, 165, 91	42,28
Cypermethrin III (alpha)	163, 181, 165, 91	42,35
Cypermethrin IV	163, 181, 165, 91	42,49
Deltamethrin	181, 253, 251, 255	46,25
Indoxacarb	218, 150, 203, 264	46,04
Lambda-cyhalothrin	181, 197, 208, 141	37,15
tau-Fluvalinate I	250, 252, 181, 251	44,45
tau-Fluvalinate II	250, 252, 181, 251	44,69
Trinexapac-ethyl	151, 224, 251, 95	19,46

Table 2
Analyte depended parameters for the analysis of pesticide residues in honey with LC-MS/MS.

Analyte	Ionisation mode	Declustering potential (V)	Multiple reaction monitoring 1 (*m*/*z*)	Collision energy (V)	Multiple reaction monitoring 2 (*m*/*z*)	Collision energy (V)
2,4-D	ESI -	−50	219 > 161	−16	219 > 125	−36
Amidosulphuron	ESI +	50	370 > 218	20	370 > 261	35
Aminopyralid	ESI +	50	207 > 189	25	207 > 161	40
Azoxystrobin	ESI +	50	404 > 372	21	404 > 344	35
Clopyralid	ESI +	50	192 > 146	46	192 > 110	46
Cyproconazole	ESI +	50	292 > 70	33	292 > 125	37
Dicamba	ESI -	−50	221 > 177	−10	219 > 175	−8
Dimethachlor	ESI +	50	256 > 224	20	256 > 148	35
Dimethoate	ESI +	50	230 > 125	29	230 > 199	15
Fenoxaprop-p-ethyl	ESI +	50	362 > 288	28	362 > 121	40
Fenpropidin	ESI +	50	274 > 147	40	274 > 86	40
Florasulam	ESI +	50	360 > 129	30	377 > 129	30
Fludioxonil	ESI -	−50	247 > 180	−42	247 > 126	−50
Fluoxastrobin	ESI +	50	459 > 427	30	461 > 429	25
Flutriafol	ESI +	50	302 > 123	39	302 > 109	43
Fuberidazole	ESI +	50	185 > 157	40	185 > 65	50
Glyphosate	ESI -	−50	168 > 63	−20	168 > 150	−16
Imazalil	ESI +	50	297 > 159	31	299 > 161	29
Imidacloprid	ESI +	50	256 > 209	21	256 > 175	19
Iodosulfuron-methyl	ESI +	50	508 > 167	20	508 > 235	30
MCPA	ESI -	−50	199 > 141	−20	201 > 143	−20
Mefenpyr-diethyl	ESI +	50	390 > 373	10	390 > 327	20
Pencycuron	ESI +	50	329 > 125	26	329 > 218	24
Picloram	ESI -	−50	239 > 195	−15	241 > 197	−15
Pinoxaden	ESI +	50	401 > 317	32	401 > 57	45
Prochloraz	ESI +	50	376 > 308	20	378 > 310	20
Propaquizafop	ESI +	50	444 > 371	24	444 > 100	24
Propiconazole	ESI +	50	342 > 159	43	342 > 69	33
Propoxycarbazone	ESI +	50	416 > 399	15	416 > 199	25
Prothioconazole	ESI -	−50	342 > 100	−32	342 > 125	−38
Pymetrozine	ESI +	50	218 > 105	25	218 > 51	75
Pyroxsulam	ESI +	50	435 > 195	30	435 > 124	70
Quizalofop-p-ethyl	ESI +	50	373 > 299	30	373 > 271	40
Spiroxamine	ESI +	50	298 > 144	29	298 > 100	49
Sulfosulfuron	ESI +	50	471 > 211	20	471 > 261	30
Tebuconazole	ESI +	50	308 > 70	39	308 > 125	47
Thiacloprid	ESI +	50	253 > 126	29	253 > 99	57
Triadimenol	ESI +	50	296 > 70	15	298 > 70	15
Triasulfuron	ESI +	50	402 > 167	25	402 > 141	30
Tribenuron-methyl	ESI +	50	396 > 181	20	396 > 155	20

glyphosate, dicamba and picloram for which the LOQ was 0.050 mg kg^{-1}.

3.2. Analysis of the honey samples

The amounts and composition of the pesticide residues found in the honey samples differed between years (Table 3). The agricultural practices generally do not vary so much, but the need for different kinds of pesticides can vary widely from year to year. The proportions of samples with at least traces of any particular pesticide were comparable, being 78% in 2013 and 63% in 2014 ($Chi2 = 0.16$; $df = 1$; $p = 0.69$), but the composition and the average cumulative amount of chemicals per sample was significantly higher in 2013 than in 2014 ($KW{-}H\,(1; 23) = 5.9$; $p = 0.015$) (Fig. 1). In 2013, five different compounds were found in the honey samples; herbicides formed a major part: clopyralid was found in 64% of samples (twice above MRL) and glyphosate in 21% (twice above MRL), whereas all glyphosate was always accompanied by clopyralid. The other compounds found in the samples in 2013 were insecticides: dimethoate, thiacloprid and tau-fluvalinate, all amounts

Table 3
The concentrations (μg kg^{-1}) of pesticide residues found in honey samples in Estonia 2013–2014.

Honey sample	Year	% of oilseed rape in foraging range	Herbicide			Fungicide		Insecticide		Insecticide, acaricide	Gross amount	No. of different compounds	
			Clopyralid	2,4D	Glyphosate	Tebuconazole	Azoxystrobin	Dimethoate	Thiacloprid	Tau-fluvalinate		Total	>LOD
1	2013	3.4	n.d.	n.d.	n.d.	n.d.	n.d.	(4)	n.d.	n.d.	4	1	0
2	2013	5.7	**272**	n.d.	14	n.d.	n.d.	n.d.	n.d.	n.d.	286	2	2
3	2013	6.2	48	n.d.	**56**	n.d.	n.d.	n.d.	n.d.	n.d.	104	2	2
4	2013	12.1	29	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	29	1	1
5	2013	10	29	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	29	1	1
6	2013	9.2	30	n.d.	n.d.	n.d.	n.d.	n.d.	(1)	n.d.	31	2	1
7	2013	12.9	(6)	n.d.	**62**	n.d.	n.d.	n.d.	n.d.	n.d.	68	2	1
8	2013	9.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
9	2013	9.1	27	n.d.	n.d.	n.d.	n.d.	(5)	n.d.	(1)	33	3	1
10	2013	14	**91**	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	91	1	1
11	2013	8.6	16	n.d.	n.d.	n.d.	n.d.	(1)	n.d.	n.d.	17	2	1
12	2013	5.1	n.d.	n.d.	n.d.	n.d.	n.d.	(1)	n.d.	n.d.	1	1	
13	2013	9.2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
14	2013	9.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
Average (μg kg^{-1})		**8.86%**	**60.9**		**44**			**2.8**	**1**	**1**	**21.2**		
15	2014	0	n.d.	n.d.	n.d.	(2)	n.d.	n.d.	n.d.	n.d.	2	1	
16	2014	8.6	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	14	n.d.	14	1	1
17	2014	1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	13	n.d.	13	1	1
18	2014	3.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
19	2014	0	n.d.	n.d.	n.d.	(5)	n.d.	n.d.	(9)	n.d.	14	2	
20	2014	2.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
21	2014	8.8	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
22	2014	2.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
23	2014	11.6	n.d.	n.d.	(9)	n.d.	n.d.	n.d.	n.d.	n.d.	9	1	
24	2014	12.3	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
25	2014	8.9	n.d.	(2)	n.d.	n.d.	n.d.	n.d.	(5)	(7)	14	3	
26	2014	13.3	n.d.	n.d.	n.d.	n.d.	n.d.	(1)	n.d.	n.d.	1	1	
27	2014	11.6	n.d.	n.d.	n.d.	n.d.	n.d.	(2)	n.d.	(6)	8	2	
28	2014	1.8	n.d.	n.d.	n.d.	n.d.	n.d.	(3)	n.d.	n.d.	3	1	
29	2014	5.9	n.d.	n.d.	n.d.	(5)	n.d.	n.d.	(7)	(6)	18	3	
30	2014	5.4	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
31	2014	8.7	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.		
32	2014	3.5	n.d.	n.d.	n.d.	n.d.	**31**	(3)	n.d.	n.d.	34	2	1
33	2014	4.9	n.d.	n.d.	n.d.	n.d.	n.d.	(3)	n.d.	(8)	11	2	
Average (μg kg^{-1})		**6.08%**		**2**	**9**	**4**	**31**	**2.4**	**9.6**	**6.8**	**11.8**		
% of samples	**2013**		**64**	**0**	**21**	**0**	**0**	**29**	**7**	**7**	**78**		
	2014		**0**	**5**	**5**	**16**	**5**	**26**	**26**	**21**	**63**		

() The numbers in parenthesis represent values under the limits of detection (LOD). The numbers in **bold** represent values above the maximum residue limits (MRL).

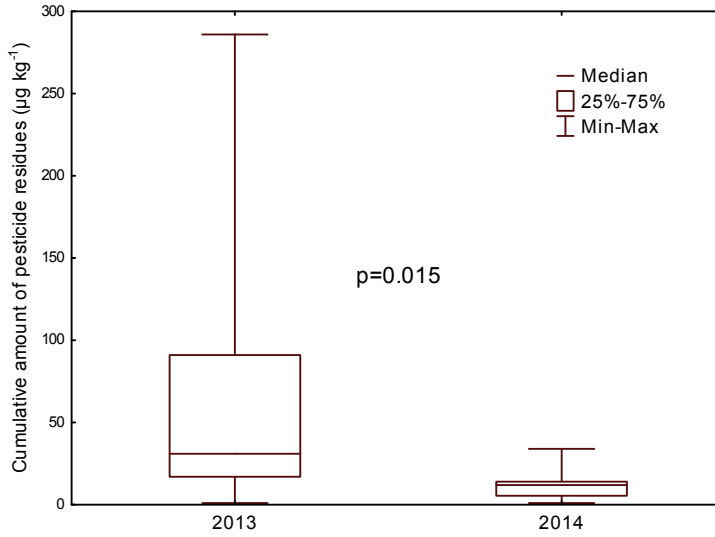

Fig. 1. Gross amount of pesticide residues calculated over all positive samples in 2013 and 2014. The Kruskal-Wallis test was used to compare median values.

remained below LOD. In 2014, however, seven different compounds were found, of which two were herbicides (2,4D and glyphosate), two were fungicides (tebuconazole and azoxystrobin) and the same three insecticides were present as in the previous year. In 2014, the amounts of pesticide residues found were much lower than in 2013, remaining between 1 and 9 μg kg^{-1} staying below the LOD, except in one sample where the fungicide azoxystrobin was found in a concentration of 31 μg kg^{-1} which is also above the MRL. In both years, the numbers of different compounds per sample stayed between 0 and 3. Although some detected pesticide residues exceeded the MRL set in Europe, these amounts still do not pose a health risk to honey consumers since the numbers remain far below the hazard index (Juan-Borrás et al., 2016). The MRLs of most pesticide residues in honey are fixed according to their lowest detection level, which means that the compounds are not allowed to contaminate the honey.

The Spearman rank order correlation did not reveal significant correlations between the cumulative amount of pesticide residues and the proportion of cultivated land ($p = 0.17$) or the proportion of oilseed rape ($p = 0.15$) in the territory within a 2 km foraging radius of honey bees. However, all except two of the pesticide residues found in the honey samples can be related to oilseed rape (Estonian Agricultural Board, 2017), which indicates the high spreading capability of pesticide residues from the fields as a potential source of contamination. The herbicide 2,4D is not allowed for weed control in oilseed rape fields, and in our study it was found only once in concentration below LOD. The herbicide clopyralid is allowed for spraying on rape plants until the formation of flower buds. Glyphosate is used before the germination of rape seed or as pre-harvest treatment against many weeds. These two herbicides, however, are commonly used in agrotechnology of many different crops. In addition, glyphosate is also sprayed to combat herbaceous grass during summer maintenance of larger roads, in greenery works in towns and cities, and also by the owners of private gardens (Estonian Agricultural Board, 2017). This means that there are several routes for glyphosate to end up in nectar collected by honey bees. The two fungicides (azoxyztrobin and tebuconazole) found in this survey are commonly used on oilseed rape against a complex of fungal diseases, and both are allowed to be sprayed during the whole flowering period. These preparations are only meant for professional pesticide users. The insecticide dimethoate is not allowed for controlling insect pests in oilseed rape cultivation in Estonia. However, this is a highly effective compound and is often used on other crops. Thiacloprid is a systemic insecticide, which is allowed to be sprayed until the full flowering of oilseed crops. The systemic nature of this compound allows it to persist in plant tissues for a long time. It is transmitted from leaves to nectar and pollen, and is thus easily attainable for foraging bees. Tau-fluvalinate, a contact insecticide, is also allowed to be sprayed against oilseed rape pest insects during flowering. Tau-fluvalinate is considered to be relatively safe for bees due to its high value of LD_{50}, which makes it possible to use the same active ingredient as varroacide inside honey bee hives. Therefore there are two different routes for how tau-fluvalinate can end up in honey (Tremolada et al., 2011), unfortunately we are not able to distinguish between them.

Honey as a product contains surprisingly few pesticide residues compared to bee bread or pollen (Thompson et al., 2014). Pesticide residues in different matrixes differ in their chemical composition and physical characteristics. Fat or lipid soluble compounds tend to contaminate wax, whereas water-soluble compounds are more readily found in nectar or honey. Besides contaminated nectar, honey contamination may also occur via translocation of the compounds from comb wax to honey (Kochansky et al., 2001; Tremolada et al., 2004).

The relatively large areas with natural vegetation, and the low amounts of pesticides used in Estonian agriculture (Eurostat, 2015) has shaped the notion that the bee forage environment should be unpolluted in Estonia and probably also in other Nordic countries. Our results, however, suggest the situation may be of concern. Despite the general low input of pesticides compared to the average usage over the European countries (Eurostat, 2015), some compounds found in honey samples exceeded the MRL. On the background of landscape characteristics, this might arise from relatively

homogeneous land cover type – in Estonia, as in Ireland and the United Kingdom, the landscape in 2015 is dominated by larger areas composed of the same land cover type, also the number of structural green elements in the landscape is small (Eurostat, 2015). Larger forest areas may serve as barriers for bees, for instance. Forests have been shown to negatively affect bumble bees with larger foraging territories (Diaz-Forero et al., 2011). Such barriers may concentrate bees on other land, thus increasing the risk of forage on polluted plants. Honey bees prefer to forage in larger open areas rich in flowers, and flowering crops make up an important part of the forage. Since it is one of the most profitable crops, oilseed rape crops are common in crop rotations: covering 15% and 11% of total cultivated land in 2010 and 2015 accordingly (Statistics Estonia, 2012).

In northern regions, the most common group of pesticides sold are herbicides: these comprise more than 70% of pesticides sold in Estonia (Eurostat, 2015). The higher amounts of herbicide active ingredients needed for effective treatments compared to insecticides, for instance, may also be one reason why herbicide residues in particular were higher in our samples. The amounts of herbicides used on fields may differ from year to year depending on the weather conditions throughout the spring and summer. The amounts of herbicides sold in Estonia were higher in 2013 compared to 2014 (Eurostat, 2015) and this appears to have been reflected in our honey samples. Although pesticide residues may be retained in soils from the previous year or even from treatments made decades ago (Hilber et al., 2008; Lozowicka et al., 2016), the authors believe this probably did not affect our results because the samples with higher concentrations in 2013 did not show higher residue level in 2014. Most of the locations sampled in 2013 were also sampled in 2014. We suppose that in those cases where we found herbicide residues higher than the MRL, the bees must have foraged on recently treated fields. For instance, glyphosate residues may remain very high in nectar for up to seven days after treatment, as demonstrated by Thompson et al. (2014). Glyphosate-based herbicides are the most common herbicides worldwide. Moreover, its usage nowadays has gone beyond pest control purposes – being more of an agricultural instead of a pest management tool (Steinmann et al., 2012). We believe that this is something to consider for reducing the levels of pesticide residue found in food: by excluding the routine spray applications and retaining the weed management purpose of glyphosate, one could facilitate a less polluted environment.

The concentrations of all residues found from honey samples in this study remained below the lethal dose to honey bees. LD_{50} is measured for 2,4D was 0.0115 mg bee^{-1} (Extension Toxicology Network, 1996), clopyralid >100 μg bee^{-1} (Dow AgroSciences, 2007) and glyphosate 100 μg bee^{-1} (Thompson et al., 2014), tebuconazole 83 μg bee^{-1}, azoxystrobin 200 μg bee^{-1}, dimethoate 0.11 μg bee^{-1}, thiacloprid 27.89 μg bee^{-1}, and tau-fluvalinlate 45 μg bee^{-1} (Sanchez-Bayo and Goka, 2014). This means that the concentrations found are definitely below acute lethal dosages, although sub-lethal effects cannot be excluded when considering that at least nurse bees consume the contaminated food until they produce the royal jelly, and also larger larvae are fed with nectar and pollen collected by foragers.

4. Conclusion

Our results demonstrate that intensively treated oilseed rape fields can be a source for pesticide residue contamination in honey, however no direct correlation was found. We believe that pesticides escape from fields over larger neighbouring areas with wild vegetation and contaminate the nectar of wild plants. Our study indicates that most of the agrochemical residues in Estonian honey can originate from oilseed treatments, however the same active ingredients are used for different crops, which is why no direct references can be made. The compounds that were represented in the highest amounts belonged to herbicides, the most frequently used pesticide group in Northern European climatic conditions. In the context of honey as human food, the concentrations of pesticide residues do not pose any health risk to consumers, although in some cases the levels detected exceeded the MRLs. Concerning the health of bees, the residues remained below acute lethality, however some sub-lethal effects cannot be excluded.

Author contribution

RK, RR, PP, IK, MM, HV conceived and designed the study, RK, RR, PP, IK collected data, VB, IP carried out pesticide analyses, RK; RR; IK; PP analysed the data, RK, RR, VB, IP, MM, HV, IHW wrote the paper, all authors read and approved the paper.

Conflict of interest

The authors declare no competing financial interest.

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III

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FIELD RELEVANT CONCENTRATIONS OF FUNGICIDE AND AN INSECTICIDE ARE AFFECTING HONEY BEE (APIS MELLIFERA) QUEENS.

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FIELD RELEVANT CONCENTRATIONS OF FUNGICIDE AND AN INSECTICIDE ARE AFFECTING HONEY BEE (*APIS MELLIFERA*) QUEENS

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Abstract

Various pesticide residues can be found from different bee colony components. Honey bee (*Apis mellifera* L.) queens receive non-contaminated food from nurse bees. Many studies show that different pesticide residues can be found from honey bee wax. Still, little is known how field realistic concentrations of lipophilic pesticides in wax affect developing honey bee queens. We investigated the impact of field relevant concentrations of the EBI fungicide tebuconazole, the insecticide tau-fluvalinate and their mixtures on developing honey bee queens during two consecutive years. Queen cell acceptance decreased due to tebuconazole, but both single compounds increased the queen weight. Besides, the interaction of these two compounds generated antagonistic effects. Additionally, the magnitude of the effects differed by years. Our findings suggest that sublethal pesticide concentrations in wax can still affect honey bee queens. This may lead to undesirable changes in honey bee colonies.

Keywords: Insecticide/tebuconazole/sublethal effects/wax/queen

1. INTRODUCTION

In honey bee (*Apis mellifera* L.) colony the honey bee queen is the most principal member. The aim of a mated queen is to encourage colony development and survival via laying eggs (Milchreit et al., 2016). During their lifetime queens are receiving pure royal jelly (RJ), excreted by nurse bees (Haydak, 1970). Different studies show that nectar, pollen and beebread, each being a food source for nurses, can be contaminated by various pesticides (Chauzat et al., 2006; Karise et al., 2017; Mullin et al., 2010; Škerl et al., 2009).

Honey bee queens are pretty well protected from xenobiotic compounds, but there is still always persistent threat to pesticide exposure. In addition to nectar, pollen and beebread, various pesticide residues have been detected in sampled honey bee wax (Chauzat and Faucon, 2007; Mullin et al., 2010; Ravoet et al., 2015). Studies have shown that insecticides used in fields and in apiculture are easily absorbed into honey bee wax-(Chauzat and Faucon, 2007; Tsigouri et al., 2004), which also might affect the developing larvae. Besides insecticides, which basically are the same compounds as agricultural insecticides, residues of agriculturally important fungicides like tebuconazole have been found in beeswax (García et al., 2017; Harriet et al., 2017; Niell et al., 2014).

Furthermore, some fungicides have been shown to synergistically increase the toxicity of certain insecticides (Johnson et al., 2013; Raimets et al., 2018). Honey bees use three different physiological systems to detoxify xenobiotics in their organisms, among these the cytochrome P450 monooxygenases play the most important role (Claudianos et al., 2006). It has been shown that EBI fungicides inhibit cytochrome P450 functioning and thus the insecticide toxicity will increase in insects (E.d et al., 1995; Pilling and Jepson, 1993). Besides synergism, the pesticide mixtures may also induce additive or antagonistic effects in insects (Cedergreen, 2014; Raimets et al., 2018).

Different studies have focused on pesticide residue impacts on honey bee workers, while queens have received little attention. The long-term effects of pesticide residues in wax can have negative effect on queen larval development. An important yet poorly understood aspect in this regard is the potentially adverse effect of different lipophilic pesticides in beeswax on developing honey bee queens, as well as queen performance during the adult stage.

In this study, we hypothesised firstly, that even low concentrations of pesticides in beeswax adversely affect developing honey bee queens; and secondly that tebuconazole as an EBI fungicide causes synergism when in mixture with the pyrethroid tau-fluvalinate. The aims thereafter are i) to monitor the effect of tebuconazole and tau-fluvalinate on development of queens from grafting them to contaminated wax until their acceptance to new colonies as laying queens; and ii) to find possible synergistic effects of tebuconazole and tau-fluvalinate.

2. MATERIAL AND METHODS

2.1. Insects used

Experiments were conducted in two consecutive years (2017, 2018). All the honey bees (*A. mellifera ligustica*) used in the experiment originated from a single apiary (OÜ R-honey) located in Eastern Estonia. Queens used in the experiment were all grafted from single queen (one-day old) larvae. Two queenless and equally sized colonies (10 langstroth frames) full of nurse bees were used as cell builders. The colonies were fed *ad libitum* with 50% sucrose solution (1:1 water:sugar) to promote queen cell acceptance.

2.2. Exposure to agrochemicals

The wax obtained from a local organic beekeeping operation was used in making queen cell cups. The active ingredients tau-fluvalinate (purity 98,7%) and tebuconazole (purity 99,3%), were both purchased from Sigma Aldrich. These pesticides were dissolved, alone and in combination, in acetone and incorporated into molten wax. In control group the queen cell cups were made similarly but without pesticides. Concentrations of pesticides used were based on findings from Estonian bee products by Raimets R (unpublished) and are similar to those found in other studies (Chauzat et al., 2011; Fulton et al., 2019). Pesticide treatments mixed into wax were: tebuconazole 412 μg kg^{-1} (2017, 2018); tau-fluvalinate 15 μg kg^{-1} (2017) and 446 μg kg^{-1} (2018); tebuconazole 412 μg kg^{-1} + tau-fluvalinate 15 μg kg^{-1} (2017); and tebuconazole 412 μg kg^{-1} + tau-fluvalinate 446 μg kg^{-1} (2018). We used two different sublethal tau-fluvalinate concentrations due to the great variability of the concentrations found from bee products. Tau-fluvalinate's LD_{50} (measured by

topical treatment) for honey bees has been determined to be 9.45 μg/bee (equals to 9450 μg kg^{-1}) (Johnson et al., 2006) and thus both concentrations used must have fitted into the sublethal dose range even if the bees had adsorbed all of it from wax. Immediately after mixing the pesticides into molten wax, queen cell cups were made using special wooden dowels to shape the cup. It is common in beekeeping practises to make queen cell cups from molten wax (Buechler et al., 2013).

2.3. Monitoring of queen development

Development of honey bee queen larvae, the weight of newly hatched queens, mating success and acceptance to new colonies were monitored. Honey bee larvae (1 day old) were taken from a single colony and transplanted into the queen cell cups, using a special spatula tool. The larvae were randomly distributed between treatments and the numbers of larvae grafted and queens left by the time of hatching are presented in Table I. Queen cell cups containing the transplanted one-day old larvae were placed into two equally sized queenless colonies for cell cup build-up. The queen cell cups from control and tebuconazole treatment groups were allocated into one and cups from tau-fluvalinate and mixture groups into another nursery hives on same langstroth frame. Both colonies were full of nurse bees to promote queen cell acceptance. After 24 hours, it was determined whether the nurse bees have accepted the cells and started feeding the larvae with RJ or not. On the fifth day post-transplant, after the accepted cells had been sealed by worker bees, the cells were relocated to an incubator (SANYO MIR – 154) where the ambient temperature was constantly 34.5 °C and RH 60%. On the tenth day, the queen cells were caged, and two days later newly emerged queens were weighed.

After weighing, the young and indivudally numbered virgin queens were introduced one by one into small four-frame mini mating hives to observe their reproductive performance. Each mini-hive was filled with worker bees and taken to the mating yard. The hives were equipped with sugar candy "Bee fonda" (Lyson) and bees had constant access to food. Two weeks after the introduction all mini-hives were inspected to determine whether the queens had started laying eggs to assess the success of mating. Mated queens were removed from mating hives and transferred into new full size colonies to observe the acceptance by worker bees.

Table I. The numbers of honey bee queen larvae and young queens in experiment

Treatment	No. of eggs grafted		No. of hatched queens	
	2017	2018	2017	2018
Control	15	25	13	20
Tebuconazole	15	25	11	7
Tau-fluvalinate	15	25	12	20
Mixture	16	25	11	14

2.4. Pesticides residues in pupal queens

To investigate whether pesticides from wax can translocate into developing queens, additional queen cell cups were made in similar way and treatments as described above. When the accepted queen cell cups were sealed by nurse bees, they were transferred into the incubator. Two days before adult emergence, the queen cells were put into a freezer (-20 °C), and pooled samples of pupae from each treatment were sent to a laboratory (Institute of Food Safety, Animal Health and Environment "BIOR") for pesticide analysis. In order to obtain a minimal critical mass for pesticide analyses the individual queen pupae were pooled to one sample consisting of 8 pupae in each treatment group.

2.5. Pesticide residue analyses from queen pupae

Tau-fluvalinate and tebuconazole residues from honey bee queen were analysed in Latvian Laboratory BIOR (Institute of Food Safety, Animal Health and Environment). The exact analysis UHPLC-MS/MS was performed using an Ultimate 3000 high performance liquid chromatograph (Thermo, USA) coupled to TSQ quantiva tandem mass spectrometer (Thermo, USA). The details of materials and performing of the analyses are described in supplementary information (SI 2.5).

2.6. Statistical analyses

Statistical analyses were performed using the program STATISTICA (version 13). Chi-square test was used to examine the effect of treatments on queen cell acceptance, rate of adult emergence, and mating success. To assess the effect of treatments on queen weight, we used the two-way full factorial analysis of variance (ANOVA) with *post-hoc* Tukey test (see more detailed in SI 2.6). All analyses were performed separately

by 2017 and 2018. To test differences in queen weights between two experimental year one-way analysis of variance (ANOVA) was used. Results were considered statistically significant when $P<0.05$.

Treatment effect sizes on queen weight were calculated according to formula:

$$\text{Effect Size} = \frac{[\text{Mean of experimental group}] - [\text{Mean of control group}]}{\text{Standard Deviation}}$$

3. RESULTS

The results of our study revealed no detectable pesticide residues in pupal queens due to the treatments. However, we detected tau-fluvalinate residues from pupae of tebuconazole treatment group (Table II).

The results of our study show that queen cell acceptance was high, between 80% and 90%, and there were no significant differences between treatment groups $P(\chi 2 > 0.44) = 0.93$ (Figure 1) in 2017. In 2018, queen cell acceptance was similarly high in control and despite of much higher concentration in tau-fluvalinate groups. However, it was significantly lower (acceptance 30%) in the tebuconazole treatment group $P(\chi 2 > 24.38) < 0.001$. Further observation of developing queen larvae indicated that the treatments did not affect the proportion of hatched queens. The reproductive performance of emerged queens (egg laying, acceptance into new colonies) was not affected by the treatments.

Our study revealed that both tebuconazole and the lower concentration of tau-fluvalinate increased the honey bee queen weight significantly in 2017 ($F(3;43)=4.99$; $p=0.005$), whereas the effect sizes were 0.89 for tebuconazole and 1.24 for tau-fluvalinate (Figure 2). However, in 2018, tebuconazole had no impact, but higher concentration of tau-fluvalinate again increased the queen weight significantly ($F(3;57)=3.57$; $p=0.020$). Despite the higher concentration of tau-fluvalinate, the effect size (0.72) was smaller. It is also important to note that in 2018 control queens weighed significantly less than queens in 2017 ($F(1,106)=38.20$; $p<0.001$).

Concerning possible synergistic increase of effects of tebuconazole and tau-fluvalinate, we did not reveal it either by queen cell acceptance, further development of queens or weight of newly emerged queen (Figures 1 and 2). Rather it seems that these two pesticides may have performed

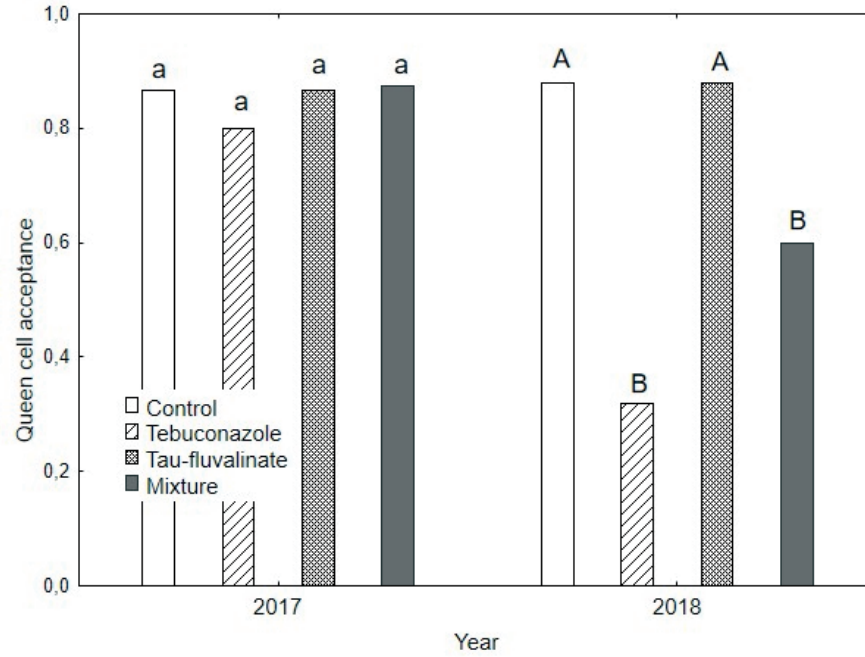

Fig. 1. Queen cell acceptance (%) by different treatment groups during 2017 (N=15 for each treatment except the Mixture, where N=16) and 2018 (N=25 for each treatment). Different letters above the boxes indicate statistically significant differences ($p<0,05$).

Table II. Pesticide residues found from honey bee queen pupae. Abbreviations: Mix 1 – tebuconazole (412 µg kg^{-1}) + tau-fluvalinate (15 µg kg^{-1}): Mix 2 – tebuconazole (412 µg kg^{-1}) + tau-fluvalinate (446 µg kg^{-1}). DL – detection limit

Treatment	Tebuconazole (µg kg^{-1})	Tau-fluvalinate (µg kg^{-1})
Control pupae	<DL	<DL
Tebuconazole pupae (412 µg kg^{-1})	<DL	193±97
Tau-fluvalinate pupae (15 µg kg^{-1})	<DL	<DL
Tau-fluvalinate pupae (446 µg kg^{-1})	<DL	<DL
Mix 1	<DL	<DL
Mix 2	<DL	<DL

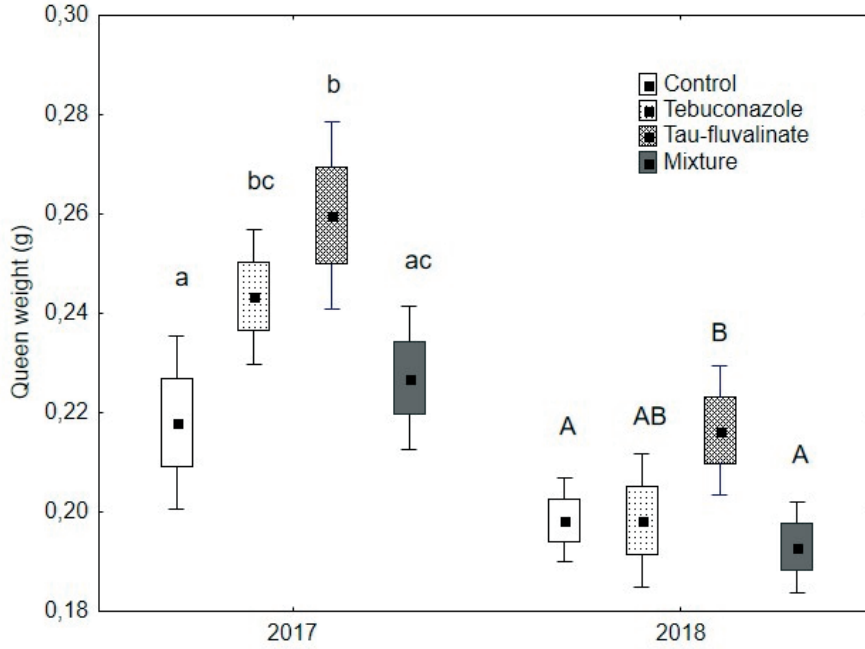

Fig. 2. Mean ±SE (dots and boxes) and 95% confidence intervall (whiskers) of newly emerged queens´ weight in 2017 and 2018. Different letters in the boxes indicate statistically significant differences between treatment groups ($p<0.05$). The numbers of queens in 2017 were: control 13, tebuconazole 11, tau-fluvalinate 12 and Mixture 11; and in 2018: control 20, tebuconazole 7, tau-fluvalinate 20 and Mixture 14.

antagonistic effects on each-other. Tau-fluvalinate might have changed the effect of tebuconazole even milder when in mixture (acceptance 60% instead of 30%). However, by the queen weight tebuconazole seems to inhibit the effect of tau-fluvalinate. Interaction effect of tau-fluvalinate and tebuconazole was statistically significant in 2017 ($F(1,43)=11.87$; $p=0.001$). Same tendency was observed in 2018, however the interaction effect was not statistically significant ($F(1,57)=3.32$; $p=0.074$).

4. DISCUSSION

This study provides novel insights into the understanding of how small amounts of pesticide residues in wax can affect honey bee queens. Results revealed that the used concentrations of single pesticides in wax had

a significant effect on queen weight and acceptance, but the effects of the mixture were antagonistic.

4.1. Queen cell acceptance

The queen cell acceptance was prohibited by the fungicide, however it appeared in only one year. Despite the fact that the queen cell cups of control and tebuconazole treatment groups were attached to the same langstroth frame and inserted simultaneously into the same colony for build-up, cell acceptance was significantly lower in the tebuconazole treatment group. We suppose this might be because of any disturbancies in hormonal systems of the larvae. As shown by Jiang He et al., (2016) starving honey bee larvae produce the volatile (ectohormone) pheromone (E)-β-ocimene to attract nurse bees to feed them. They also showed that three genes (*llp-like, fps and aatc-like)* responsible for (E)-β-ocimene production were more highly expressed in young (two-day old) queen larvae (He et al., 2016). Our queen larvae were exactly two days old at the time of acceptance monitoring. Queen cell acceptance by nurse bees in tebuconazole treatment group was probably lower due to fact that tebuconazole might have impaired the larval hormonal system and thus they probably were unable to produce volatile pheromone (E)-β-ocimene. A possible reason why this effect was observed only in one year and not in another might be different food availabilities between 2017 and 2018. It may be that in extremely dry summer of 2018 the honey bees did not find enough pollen to satisfy the nutritional needs of the nurse bees and thus the larvae may have suffered poor nutrition. Although the nurse colonies were fed *ad libitum* with 50% sucrose solution to promote queen acceptance, this could not satisfy their protein needs. We suppose that in this condition the mild effect of tebuconazole could have emerged at recognizable level. Another -azole type fungicide propiconazole has been shown to affect larval development time and survival in *Mamestra brassicae* L (Johansen et al., 2007). It seems that the first instar larvae are most acceptable to small disturbances, since no further effects were observed throughout the queen larval or adult development. No significant changes were observed on hatching rate, mating confirmation or mated queen acceptance to new colonies.

4.2. Weight of queens

The weight of newly emerged queens was also affected by treatments, however here the effect came mostly from tau-fluvalinate. The detected

increase in queen weight due to tau-fluvalinate exposure is in contrast with some other studies. Rangel and Tarpy (2015) saw no change in queen weight but observed slightly larger head and thorax measures, when the queens had been reared on queen cell cups contaminated with miticides (combination of tau-fluvalinate and coumaphos). Pettis et al. (2004) showed that coumaphos in wax of queen cell cups decreased queen acceptance substantially, and emerging queens weighed significantly less than controls. Similar weight decrease is shown by Haarmann et al., (2002) after rearing queens in hives with varroacide treatments. However, in those studies the pesticides concentrations used were much higher than in the present study. In case of very low doses, as was matter in our study, it has been observed that pesticides can cause positive instead of negative effects. Cutler and Rix (2015) discuss the hormesis in bees. Whether hormetic effect is positive or negative to queens remains unknown yet. In general, beekeepers prefer heavier queens assuming they would lay more eggs, but pesticide caused stimulation of any particular function might not give better fitness. In this study we did not observe any disturbances in queen performance until acceptance into new colonies. The future experiments should be prolonged to determine the long-time performance of the queens.

In the present study, we suggest that tau-fluvalinate also could have affected the queen bee endocrine system. In the bee brain, the gland *Corpora allata* is the part of endocrine system, which is responsible for juvenile hormone production (Cutler and Rix, 2015). Hormones play a key role in insect homeostasis, and thus changes in endocrine system may affect insect homeostatic mechanisms such as metamorphosis, food intake, and activity of neurons and muscles (Cutler and Rix, 2015). In light of our current study, there is the possibility that tau-fluvalinate, as a pyrethroid, affected the neuroendocrine system of developing queens, leading to changes in hormone production and queens consuming more food during larval stage.

Another important factor affecting queen weight is the age of larvae at the time of transplanting to queen cell cup. Different studies have shown that four-day old transplanted worker larvae are still able to develop into queen, although these queens were smaller in size and weighed less (Gilley et al., 2003; Weaver, 1957; Woyke, 1971). In our study, we grafted only one-day old larvae, which excludes the latter possibility.

Queens exposed to the fungicide tebuconazole showed higher body weight than control queens in 2017, but no effect was seen in 2018. In 2018 the overall queen weight also in control group was lower and the probable poor nutrition might have shaded the slight effect of this chemical on queen weight.

Lipophilic pesticides like tebuconazole and tau-fluvalinate can easily accumulate into wax (García et al., 2017; Mullin et al., 2010), and due to continuous agricultural as well as apicultural application the numbers of residues in bee matrices will increase in time. It is vital to understand whether pesticide residues from wax can be taken up by honey bees during their developmental stage. Medici et al., (2012) show that the presence of insecticides in wax negatively affects honey brood survival. However, few of the conducted studies have focused on the translocation of pesticides from wax to bees, especially to queen bees. While one study showed that nurse bees that feed on contaminated pollen and nectar produced uncontaminated RJ for honey bee queens (Chauzat and Faucon, 2007), another study showed that some pesticide residues can be found in royal jelly, though in potentially negligible concentrations (max. concentration found was 0.016%) of the original concentration fed to the nurse bees (Ravoet et al., 2015). In another an experimental study, hives were treated with known amounts of tau-fluvalinate via contaminated plywood inserts, and no residues were detected in royal jelly (Tsigouri et al., 2004).

Fulton et al., (2019) demonstrate the both adult larval honey bees can obtain measurable concentrations of fluvalinate through both contaminated wax and presence of medicaments in hives and this is dependent on exposure times (Fulton et al., 2019). As a result of wax contamination in present study, we did not find detectable amounts of used pesticides from queen pupae. The controversial finding of tau-fluvalinate in pupae from tebuconazole treatment indicates that the source of it must have been the original wax used for building queen cell cups. All the samples were marked thoroughly and double-checked, so that there cannot be any mis-labeling or mis-reporting on behalf of the lab. Also (Fulton et al., 2019) recognized that even when the wax originates from organic beekeeping operation and is thought to be clean from any pesticides, it might not be true. Tau-fluvalinate's lipophilic properties, as well as its intensive use in agriculture and apiculture, may explain the presence of its residues. Despite the fact that the wax for all treatment groups originated from the same pool, we did not see tau-fluvalinate residues in other groups. It may

be possible that we did not see a precise reflection of residues in wax due to the small volumes of materials analysed. Using larger number of samples may help to determine pesticide residues more precisely and decrease possible variation. Although no residues were found in other treatment groups, there is always the possibility for pesticide presence, as the concentrations in samples could be under the limit of detection.

4.3. Interaction of the pesticides

Tebuconazole alone has low toxicity to honey bees, yet has been shown to disturb their ability to detoxify other chemicals (Thompson et al., 2014). This means, that we expected to see either additive or synergistic effect of these two chemicals. Instead, we saw rather antagonistic outcome. Independent of the concentration of tau-fluvalinate, tebuconazole seemed to inhibit the effect of it when looking on queen weight data. The queen acceptance data thereafter shows the disappearance of the effect of tebuconazole in the presence of tau-fluvalinate. Cedergreen (2014) revealed in her meta-analysis that the occurrence of antagonistic effects by mixtures of pesticides was largely due to cholinesterase inhibitors and azole fungicides, which made up 29% of the antagonistic mixtures. There might be different mechanisms why antagonism of pesticide active ingredients occurs. For instance, when more toxic compound prohibits the feeding, the other compound will not be consumed at quantities large enough to cause any impact and as a result, the effect of both stay minimal (Raimets et al., 2018). The authors are not aware whether these two chemicals can affect the translocation of each other from wax to the larval body. The mechanisms of antagonistic effects of pesticides on honey bees should gain more scientific attention to explore the topic of pesticide hazards.

5. CONCLUSION

The present study provides information about possible disturbances of even very low concentrations of pesticides to honey bee queens, whose primary task is to generate offspring within the colony. Moreover, the co-existence of multiple pesticides in bee environment may generate unexpected results with unknown implications. We saw decreasing queen cell acceptance with tebuconazole and increasing adult queen weight due to tebuconazole and tau-fluvalinate as single compounds, however in interaction these two caused an antagonistic outcome. For bees, due to intensive farming prac-

tices and synthetic medical pesticide usage in apiculture, there is always a potential threat to simultaneous pesticide exposure. For queen rearing practices, we suggest to use newly excreted honey bee wax for performing queen cell cups in order to prevent previous wax contamination by pesticides.

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Authors contribution
RR, MM and RK designed the study and did the manuscript preparation. RR and SN collected the data. RR, RK, TK and VB analysed the data and contributed to manuscript preparation.

Conflict of interest
The authors declare that they have no potential conflict of interest in relation to the study in this paper.

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IV

Raimets, R., Karise, R., Mänd, M., Kaart, T., Ponting, S., Song, J., Cresswell, J.E. 2018.

SYNERGISTIC INTERACTIONS BETWEEN A VARIETY OF INSECTICIDES AND AN EBI FUNGICIDE IN DIETARY EXPOSURES OF BUMBLE BEES (BOMBUS TERRESTRIS L.).

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Synergistic interactions between a variety of insecticides and an ergosterol biosynthesis inhibitor fungicide in dietary exposures of bumble bees (*Bombus terrestris* L.)

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Abstract

BACKGROUND: In recent years, concern has been raised over honey bee colony losses, and also among wild bees there is evidence for extinctions and range contractions in Europe and North America. Pesticides have been proposed as a potential cause of this decline. Bees are exposed simultaneously to a variety of agrochemicals, which may cause synergistically detrimental impacts, which are incompletely understood. We investigated the toxicity of the fungicide imazalil in mixture with four common insecticides: fipronil (phenylpyrazoid), cypermethrin (pyrethroid), thiamethoxam, and imidacloprid (neonicotinoids). Ergosterol biosynthesis inhibitor (EBI) fungicides like imazalil can inhibit P450 detoxification systems in insects and therefore fungicide – insecticide co-occurrence might produce synergistic toxicity in bees. We assessed the impact of dietary fungicide – insecticide mixtures on the mortality and feeding rates of laboratory bumble bees (*Bombus terrestris* L.).

RESULTS: Regarding mortality, imazalil synergised the toxicity of fipronil, cypermethrin and thiamethoxam, but not imidacloprid. We found no synergistic effects on feeding rates.

CONCLUSION: Our findings suggest that P450-based detoxification processes are differentially important in mitigating the toxicity of certain insecticides, even those of the same chemical class. Our evidence that cocktail effects can arise in bumble bees should extend concern about the potential impacts of agrochemical mixtures to include wild bee species in farmland.

Supporting information may be found in the online version of this article.

Keywords: bumble bees; ergosterol biosynthesis inhibitor fungicide; insecticides; synergy

1 INTRODUCTION

Recently, concern has been raised over pollinator declines in Europe and North America.[1] In some regions, beekeepers have experienced severe losses among colonies of managed honey bees (*Apis mellifera* L.)[2] and among some wild bees[3] there is evidence for extinctions[4] and range contractions.[5] Bee declines are of concern because of the valuable pollinator services that they provide to crops and wildflowers.[6,7] The declines probably have various anthropogenic causes, including the use of pesticides in intensively cultivated farmland.[8]

In farmland, pollinators may be exposed to several pesticides during their lifetime because numerous pesticide residues are present in bee forage plants[9] and in various hive matrices of managed honey bees.[10] For example, Mullin *et al.*[11] found 118 different pesticides and their metabolites among the various matrices (e.g. stored honey and bee bread) of hone bee hives. Contemporary intensive agriculture involves protecting crop plants with a variety of pesticides, including fungicides and insecticides, and bees will almost certainly encounter these residues in mixture when they forage in agrochemically treated bee-attractive crops.[12,13]

The existence of disproportionate, or non-additive, toxicity of pesticides in mixture is known as a 'cocktail effect', 'synergistic interaction',[14] or 'potentiation'.[15] Our focal example arises from the capacity of certain fungicides, which typically have low toxicity to insects, to greatly increase the toxicity of an insecticide by inhibiting the insect's capacity to metabolically degrade the insecticide. Specifically, the widely used group of fungicides known as ergosterol biosynthesis inhibitors (EBIs) are well known

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to increase toxicity to honey bees of pyrethroid insecticide in mixture.[16] However, while mixture effects have been tested widely in honey bees,[17,18] the susceptibility of wild bees to these synergistic interactions has not been fully explored. We therefore investigated the potential for an EBI fungicide, imazalil, to synergise (or, more strictly, potentiate) the toxicity to bumble bees of environmentally relevant insecticides from a varied range of chemical families, namely the neonicotinoids (thiamethoxam and imidacloprid), pyrethroids (cypermethrin) and phenylpyrazoles (fipronil).

The four focal insecticides that we studied all target the insect nervous system. The neonicotinoids block the ligand-gated ion channels of the nicotinic acetylcholine receptors. In bees, dietary exposure to neonicotinoids can impair a wide range of behavioural and life history-related characteristics[19] including homing behaviour,[20] colony performance[21] and foraging activity.[22] The pyrethroid cypermethrin affects insect sodium channels[23] and has been demonstrated to affect longevity[24] and respiratory patterns[25] in bees. The phenylpyrazole fipronil blocks receptors that respond to the neurotransmitter gamma-aminobutyric acid (GABA)-gated chloride channels in the insect central nervous system and can affect longevity in bees.[26]

We chose imazalil to represent the EBI fungicides. Imazalil is environmentally relevant because its residues can occur in combination with imidacloprid in fruit orchards[27] and it is water soluble, which facilitates dose preparation. In view of their low toxicity to insects in pure exposures, EBI fungicides are not considered harmful to farmland bees provided that the 'good practice' label rates and prescriptions are followed.[28] However, the EBI fungicides can detrimentally affect bees' tolerance for other pesticides because of effects on metabolic detoxification pathways. A certain degree of insecticide tolerance in bees is possible as a consequence of metabolic detoxification of the active ingredients by enzymes of the cytochrome P450 system.[17] Impairment of the P450 system by EBI fungicides can result in an increase of insecticide toxicity for bees.[29] Therefore, the principal aim of our present study was to establish the involvement of metabolic detoxification in bumble bee – pesticide interactions by testing whether imazalil synergises various insecticides representing some of the major chemical families that are widely used in farmland crop protection.

2 MATERIALS AND METHODS

2.1 Bee provenance and husbandry

Bumble bees (*Bombus terrestris* L. ssp *audax*) were purchased as boxed queen-right colonies from commercial suppliers (Koppert Biological Systems, Berkel en Rodenrijs, the Netherlands and BioBest, Westerlo, Belgium). For each of five separate experiments, adult workers were collected from a single colony under red light and individually allocated to a wooden cage (0.07 x 0.05 x 0.04 m) whose two largest faces were covered by ventilating mesh. Each cage was supplied with a small *ad libitum* syrup feeder. During experiments, the bees were kept in a semi-controlled environment (24 ± 1 °C, ~47% relative humidity and 12:12 h dim light:darkness). During experimental exposures, the caged bumble bees were fed on dose-appropriate syrup *ad libitum* and their feeders were weighed before and at the end of the experiment (after 48 h of exposure) in order to measure syrup consumption. We recorded mortality at 24 and 48 h of exposure. Bees were considered dead when they did not move their legs or antennae and did not respond to stimulation.

2.2 Exposure to agrochemicals

In order to test for synergistic interactions between the fungicide and a single insecticide, each experiment comprised four treatments: (1) undosed controls; (2) fungicide alone; (3) insecticide alone; and (4) fungicide – insecticide mixture. At the University of Exeter laboratory, we conducted four separate experiments (one per focal insecticide) in which we delivered sublethal dietary doses of the four agrochemical treatments in feeder syrup (Attraker; Koppert Biological Systems). At the Estonian University of Life Sciences laboratory, Tartu, we repeated the experiment conducted at Exeter (12 bees per treatment) with imidacloprid using both a larger number of replicates (i.e. 20 per treatment) and also the local procedures for dose preparation in order to validate the result previously obtained at Exeter. Except for the imidacloprid experiment at Exeter, each treatment was replicated in at least 20 bumble bee individuals in every experiment.

For each agrochemical, we used experimental doses (see below) that aimed to produce approximately 20% mortality in exposures to single dietary substances. The purpose of this level of dosing was both to demonstrate that the fungicide and insecticide were physiologically active in the exposed bees and also to provide enough capacity for the dietary mixture to reveal a statistically detectable synergistic interaction between the test substances, if it should exist. Specifically, if the two test substances each separately cause 20% mortality in treatment groups of 20 bees (i.e. 4 deaths per treatment), then their mixture is expected to cause 36% mortality (i.e. approximately 7 deaths) if they act independently (see Eqn 1 below) and a statistically significant non-independence (synergy) is detected when mortality exceeds 65% (13 deaths) in the mixture (see statistical testing below).

Before incorporation into diets, the active substances were dissolved initially in small volumes of acetone, which was subsequently adjusted so that syrup in each treatment group contained 1% acetone, including the undosed control diet, according to the method described by Thompson *et al.*[24] The dietary concentrations of the active substances in the feeder syrups were as follows: imazalil (Sigma Aldrich), 300 mg L^{-1}; fipronil (Sigma Aldrich, Poole, UK), 20 μg L^{-1}; thiamethoxam (Sigma Aldrich), 13 μg L^{-1}; imidacloprid (Sigma Aldrich), 500 μg L^{-1}; cypermethrin (Sigma Aldrich), 7 mg L^{-1}. The doses were established based on data from the literature and pilot experiments. The relatively high ratio of fungicide:insecticide concentrations in our diets facilitates the manifestation of synergistic interactions.[16]

2.3 Statistical analyses

We tested statistically for synergistic interactions between the fungicide and a single insecticide with a modified binomial proportion test for additivity (BPA).[38] The BPA test uses the 'Bliss independence criterion',[30] whose basis is that:

$$p_{AB}^{exp} = p_A + p_B - p_A \cdot p_B \quad (1)$$

where p_A and p_B denote the probabilities of mortality attributable to dietary substances A and B, respectively, and p_{AB}^{exp} denotes the expected probability of mortality attributable to a dietary mixture of A and B if they act independently. If p_{AB}^{obs} denotes the observed proportion of bees that die by consuming the dietary mixture of A and B, then the null hypothesis of an absence of interaction is:

$$H_0 \equiv D = \left(p_{AB}^{obs} - p_{AB}^{exp}\right) = 0 \quad (2)$$

An expression that evaluates the sampling distribution of D under H_0 as a z-score has been produced by Sgolastra *et al.*,[31]

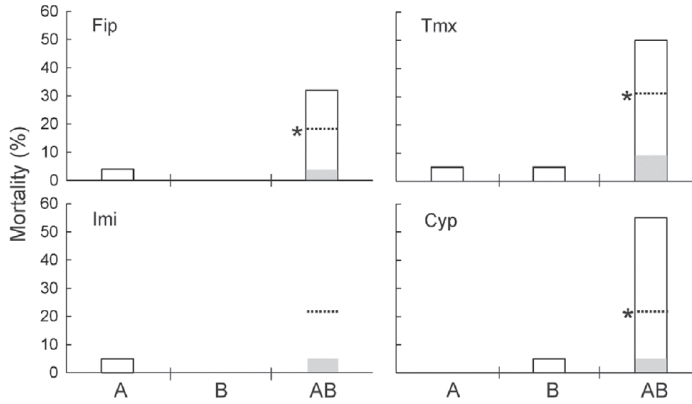

Figure 1. Mortality [proportion (%) dying] after 24 h in three exposure treatments: A, dietary imazalil; B, insecticide (Fip, fipronil; Tmx, thiamethoxam; Imi, imidacloprid; Cyp, cypermethrin); and AB, imazalil – insecticide mixture. In the AB column, the grey fill indicates the expected mortality if the components of the dietary mixture act independently (H_0) and the dashed horizontal line indicates the upper 95% confidence interval on the sampling distribution under H_0. An asterisk indicates that the mixture has produced a statistically significant synergistic effect (one-tailed binomial proportion test). A column is blank (has no bar) if no mortality occurred.

which enabled us to obtain *P*-values by approximation to a standard normal distribution. For each insecticide, BPA tests were performed separately for mortality at 24 and 48 h. For each focal insecticide, variation among treatments in feeding rate was analysed with one-way analysis of variance (ANOVA) and Tukey post hoc tests. In analysing feeding rates at 48 h, only data from bumble bees alive at 24 h were used.

3 RESULTS

No mortality was observed in any of the control exposures (undosed syrup). When mortality was the response variable, we detected synergistic interactions between imazalil and fipronil (BPA test: 24 h, $P < 0.005$; 48 h, not significant), thiamethoxam (BPA test: 24 and 48 h, $P < 0.005$) and cypermethrin (BPA test: 24 and 48 h, $P < 0.001$) (Figs 1 and 2). Dietary exposure to imidacloprid alone (500 μg L^{-1}) caused little mortality and we did not detect positive synergistic interactions between imazalil and imidacloprid in the experiment at Tartu (Figs 1 and 2). Dietary imidacloprid reduced the mortality rate resulting from dietary imazalil in the Exeter experiment (BPA test: 24 h, $P < 0.005$; 48 h, $P < 0.001$; Supporting Information Fig. S1).

Feeding rates varied among the dietary treatments (one-way ANOVA: fipronil: $F_{3,87} = 17.1$, $P < 0.001$; thiamethoxam: $F_{3,60} = 15.6$, $P < 0.001$; imidacloprid: $F_{3,73} = 5.2$, $P < 0.01$; cypermethrin: $F_{3,64} = 25.3$, $P < 0.001$) and generally dietary agrochemicals reduced syrup consumption (Tukey post hoc tests: $P \leq 0.05$; Fig. 3), but no interactions were observed between insecticides and the fungicide.

4 DISCUSSION

4.1 Synergistic effects – physiological implications

Our present study revealed that dietary exposure to the fungicide imazalil increased the toxicity to bumble bees of three out of the four insecticides that we tested, which indicates that it has the capacity to cause a positive synergistic interaction, or cocktail effect, in these insects. Our findings are consistent with those of several previous studies of the effects on honey bees of fungicides in mixture. In honey bees, prochloraz synergises both pyrethroid[32] and pyrazole[29] insecticides, and thiamethoxam (a neonicotinoid insecticide) is synergised by both tebuconazole[16] and boscalid.[13] Fungicides that synergise the toxicity of insecticides in honey bees act by inhibiting detoxification systems, such as the P450 enzyme complex.[33] Taken together with previous work, our results suggest that the P450s could play an important role in both honey bees and bumble bees in the detoxification of a chemically varied group of active ingredients from three chemical families, namely the phenylpyrazoles (i.e. fipronil), the pyrethroids (cypermethrin) and the neonicotinoids (thiamethoxam). These findings have a straightforward adaptive explanation because the season-long activity of social bees makes them forage-generalists who must subsist on nectar and pollen from a wide variety of plant species, each of whose blooming period is shorter than the lifespan of the colony. Many plants protect their pollen against consumption by non-pollinating flower visitors with secondary chemicals,[34] which vary in constitution among plant lineages. Social bees therefore have evolved to cope with a broad spectrum of plant secondary chemicals in their diet including metabolic detoxification by active enzymes (e.g. P450 systems) in the digestive tract. These considerations suggest that social bees, including bumble bees, are pre-adapted for tolerating dietary insecticides that are artificial analogues of naturally occurring plant toxins,[35] such as the nicotine- and pyrethrum-based toxicants used in the present study. It also implies that oligolectic solitary bees could be more susceptible to insecticides than their social counterparts.

Our present investigation found no evidence for a synergistic interaction during dietary exposure to a mixture of a known P450 inhibitor, imazalil, and imidacloprid in bumble bees. Similarly, previous research that exposed honey bees to imidacloprid using oral doses found little synergistic interaction with EBI fungicides.[16] Contact applications of active ingredients to the thorax of honey bees also produced very weak synergistic effects of piperonyl butoxide (PBO; another P450 inhibitor) on imidacloprid,

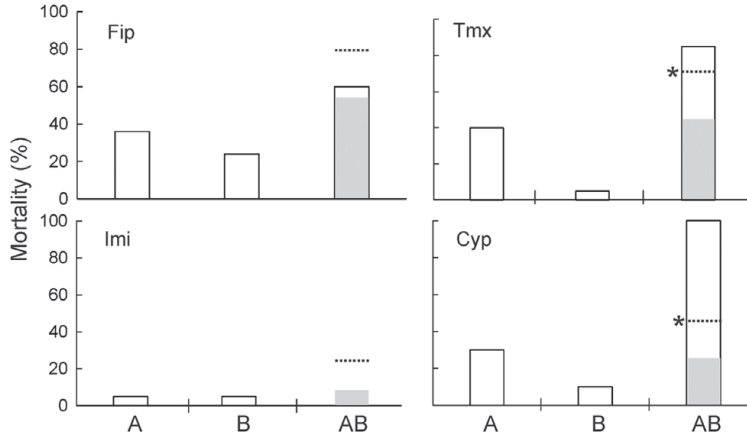

Figure 2. Mortality [proportion (%) dying] after 48 h in three exposure treatments: A, dietary imazalil; B, insecticide (Fip, fipronil; Tmx, thiamethoxam; Imi, imidacloprid; and Cyp, cypermethrin); and AB, imazalil – insecticide mixture. In the AB column, the grey fill indicates the expected mortality if the components of the dietary mixture act independently (H_0) and the dashed horizontal line indicates the upper 95% confidence interval on the sampling distribution under H_0. An asterisk indicates that the mixture has produced a statistically significant synergistic effect (one-tailed binomial proportion test). A column is blank (has no bar) if no mortality occurred.

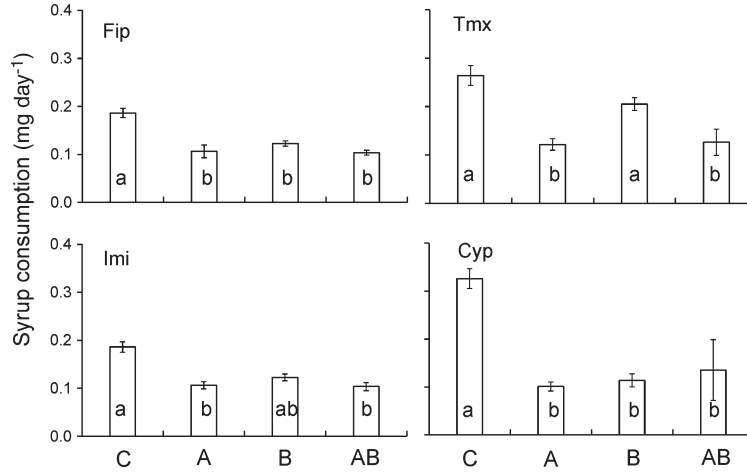

Figure 3. Variation in individual feeding rates (mg syrup consumed per bee per day) during 48 h of exposure among four dietary treatments: C, undosed controls; A, dietary imazalil; B, insecticide (Fip, fipronil; Tmx, thiamethoxam; Imi, imidacloprid; and Cyp, cypermethrin); and AB, imazalil – insecticide mixture. Among the histogram columns, different lower case letters indicate significant differences in mean feeding rate (Tukey test, $P < 0.05$). Error bars indicate 1 standard error.

even though PBO strongly synergised the toxicity of two other neonicotinoids, acetamiprid and thiacloprid.[36] Based on these results, we tentatively propose two hypotheses. First, it is conceivable that separate detoxification systems deal with imidacloprid and the other toxicants and that one hallmark of the proposed imidacloprid-specific enzyme system is insensitivity to inhibition by imazalil and PBO. However, it is unclear what detoxification enzyme could be both specific to imidacloprid and also selectively immune to interference from imazalil and PBO. Second, it is possible that imazalil suppresses the metabolic activation of imidacloprid by a P450 enzyme system. Imidacloprid has toxic metabolites, 5-hydroxyimidacloprid and olefin, that are implicated in causing mortality.[37] Disruption of metabolic activation may also explain why the synergistic effects of imazalil on fipronil that were evident at 24 h had disappeared by 48 h; specifically, inhibition of P450 oxidative enzymes may reduce the production of fipronil's highly toxic sulfone metabolite.[38] Consequently, we postulate that complex mixture effects can arise when both detoxification and metabolic activation of an insecticide are inhibited by a second active substance, such as a fungicide.

In contrast to the effects on mortality that we observed in our experiment, no synergism was detected in regard to feeding rate, although the separate exposures to the fungicide and insecticides decreased it. These results provide further confirmation of

differential sensitivity to pesticides among various endpoints such as mortality and feeding rate.[39] Despite the reductions in feeding rates caused by dietary agrochemicals, it is unlikely that any of the individuals in our experiments died from starvation within the 48-h exposure, because dosed bumble bees can live for 35 days while feeding at less than half the rate of undosed controls.[40]

We observed differences among our separate experiments in the levels of mortality caused by exposure to dietary imazalil. We expect that these differences originated in either intrinsic or environmental variation in the bumble bee colonies used, because our experiments were conducted at different times of year and for each experiment new bumble bee colonies were purchased. However, while the differences indicate that the severity of mixture effects can be expected to vary among real-world instances, it is unlikely that the existence of synergistic interactions (i.e. our main conclusion) can itself be governed by environmental influences or genetic variation among bees.

4.2 Synergistic effects – environmental relevance

Our results indicate that exposures to environmentally relevant mixtures of pesticides could be potentially harmful to wild bees even when the impacts of separate exposures to the mixture's single components are negligible. Specifically, our experiments confirm that cocktail effects arising from agrochemical pesticides are physiologically possible in bumble bees, but we recognize that further research is needed to establish their potency when bees are exposed to residues at environmentally realistic levels, which are likely to be lower than those we studied here. Thus, further empirical testing of pesticide mixtures is warranted and should be taken into account in regulations that govern the use of fungicides and insecticides in farmland.

4.3 Summary

Our present study revealed that certain insecticide – fungicide mixtures (except imidacloprid – imazalil) positively synergised the effect of the insecticide in bumble bees when assessed by levels of mortality, but not when assessed by variation in feeding rates. The efficacy of imazalil (an EBI fungicide) to synergise the toxicity of chemically varied insecticides suggests that P450 systems are involved in broad-spectrum detoxification in bumble bees. As previously found, imidacloprid alone was weakly synergised and the physiological basis of this differentiation is a target for future research. Our evidence that cocktail effects can arise in bumble bees should extend concern over the potential impacts of agrochemical mixtures to include wild bee species in farmland.

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SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

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1.13 Using respiratory physiology techniques in assessments of pesticide effects on bees

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Abstract

The determination of sub-lethal effects of pesticides on beneficial insects is challenging topic because the vast number of different possible endpoints. Traditionally measured endpoints reflect the basic outcome but do not give any information about the mode of actions or the real non-harming dosages of the studied toxicants. Physiological changes, however, reflect even small deviations from normal state. The gas exchange patterns are sensitive cues to determine the sub-lethal toxicosis in insects. Methods of respiratory physiology have been used to detect sub-lethal toxic effects of many chemicals, but information for biological preparations is also needed, especially when bees are used in entomovectoring task.

The aims of this study were i) to clarify which are the effects of three microbiological preparations on two bee species, honey bees *Apis mellifera* L. and bumble bees *Bombus terrestris* L. and ii) could we compare the effects of the same preparations on different bee species. We saw that honey bees and bumble bees react similarly on microbiological preparations, however the reaction strength differed. We found that kaolin affects the survival of bumble bees and honey bees as much as did entomopathogenic preparations, whereas pure spores of a non-hazardous fungus and wheat flour did not. Bumble bees seem to be more tolerant to microbiological preparations than honey bees.

Keywords: measuring sub-lethal effect, honey bee, bumble bee, microbiological preparation

Introduction

Pesticide residues in environment are told to be among the reasons contributing to decreasing pollinator populations.[1] Establishment of lethal dosages or concentrations to both target and non-target organisms is demanded by legislation process of pesticides, but sublethal effects have gained much less attention. However, the sub-lethal effects of pesticides may affect insects

severely through chronic stress[2] or fostering the effects of other stress factors, ultimately leading to decreasing fitness of populations.[3]

Determination of such sub-lethal changes, which cannot be captured by a human eye, might give us knowledge to explain factors leading to bee declines for both domesticated and wild bees. We know much about the concentrations of residues in soils, plant tissues, nectar and pollen,[4] however we do not know how insects cope with the residues they are constantly in contact. Talking about non-harming dosages needs clarification of real versatile dosages of an active ingredient or a preparation. The behavioural changes might not reflect the effects[5] nor the border between real harming/non-harming level of toxicants due to the buffering capacity of the organisms or the bee colonies. Molecular and cellular methods typically require killing of the study-organism. Still, some physiological mechanisms allow working with living and intact insect. Among the latter, methods of respiratory physiology determine the rates of metabolic and water loss levels, muscle activity, heart pulsation and respiratory patterns, which easily react on any changes of stress factors.[6]

Respiratory measurements are highly sensitive and reflect any minor changes in environmental or organism functioning level. Metabolic rate that is calculated based on oxygen consumption or carbon dioxide release is most commonly measured parameter. Combining it with water loss rate and respiratory patterns gives understanding that is more detailed. Already in 1991, Kestler[7] has demonstrated the changes in respiratory patterns following to sub-lethal or lethal contact of an insecticide, which targets insect nervous system. He was first who described the respiratory pattern transitions due to poisoning and also determined the pattern, which indicates irreversible toxicosis.

Beside synthetic pesticides, also different biocontrol agents are used in plant production. These preparations also need detailed information about the modes of actions, lethal or sub-lethal dosages or harmful side-effects. More-over, when microbiological preparations are to be applied to crops using bees as vectors for preparations,[8-10] the safety of bees must be guaranteed. Both honey bees and bumblebees are used in bee-vectoring task, however the sublethal effects of preparations is not clear. The aims of this study were i) to clarify which are the effects of three microbiological preparations on two bee species, honey bees *Apis mellifera* L. and bumble bees *Bombus terrestris* L. and ii) could we compare the effects of the same preparations on different bee species.

Material and Methods

Bumble bees (2 hives) were purchased from Koppert Biological systems (Berkel en Rodenrijs, the Netherlands). Honey bees (one colony) were purchased from a local beekeeper. The exact age of the bees was unknown; however, we aimed to study only forager bees, bumble bees were captured from hive entrances and honey bees were caught with in insect net after when they were flying out for forage.

We used one biofungicide Prestop-Mix, which contains spores of *Gliocladium catenulatum* J1446 strain from Verdera (Espoo, Finland), and two bioinsecticides BotaniGard containing *Beauveria bassiana* GHA strain and Met52 *Metarhizium brunneum* Strain F52 (both from Borregaard BioPlant ApS, Aarhus, Denmark) in our experiments. In addition we tested the effects of pure *G. catenulatum* spores and some inert materials used as carrier compounds in preparations: kaolin ($[Al_2Si_2O_5(OH)_4]$, particle size: 3 microns, Bang to Bonsomer Estonia (Tallinn, Estonia) and wheat flower (Tartu Mill (Tartu, Estonia) since different corn flowers are also used as carrier materials.

Bees were treated individually with any of the powders with an amount that covered the bee with a thin powder layer by shaking them tenderly in a vial containing 20 mg for honey bees and 50 mg for bumble bees. Control bees were also treated similarly in an empty vial. All bees were kept individually in plastic vials (perforated walls to allow hearing and smelling of each-other) at a temperature of 28 ˚C and RH=60% in 12:12 light:darkness regime (SANYO - Versatile

Environmental Test Chamber, MLR-351, Japan). Each bee was provided 30% sugar solution as food.

The bee survival was monitored daily until all bees were dead. Metabolic rate (MR VCO_2, ml h^{-1}) and water loss rate (WLR VH_2O, µl h^{-1}) was measured by means of LI-7000 differential CO_2/H_2O analyser (LiCor, Lincoln, NE).[11] Each individual was measured 3 hours before and 3 hours after the treatment.

For statistical analyses of data Kruskal-Wallis ANOVA (survival data) and one-way or factorial ANOVA (MR and WLR data) (α=0.05) was used. In comparison of MR and WLR change in time (control groups only) paired t-test was performed.

Results

Bumble bees lived significantly longer than honey bees in such kind of experiment (KW-H(1;80)=44.9; p<0.001). In both groups the treatment affected the longevity of bees (bumble bees: KW-H(4;97)=16.2; p<0.01, honey bees: KW-H(6;480)=152.9; p<0.001). Control and wheat flour did not affect bee survival. Surprisingly, the biofungicide Prestop-Mix affected bee survival significantly in both bee species (see also Karise et al., 2016[11]), although pure *G. catenulatum* which was tested only on honey bees did not affect it. The kaolin caused as low survival as did bioinsecticides (Figure 1).

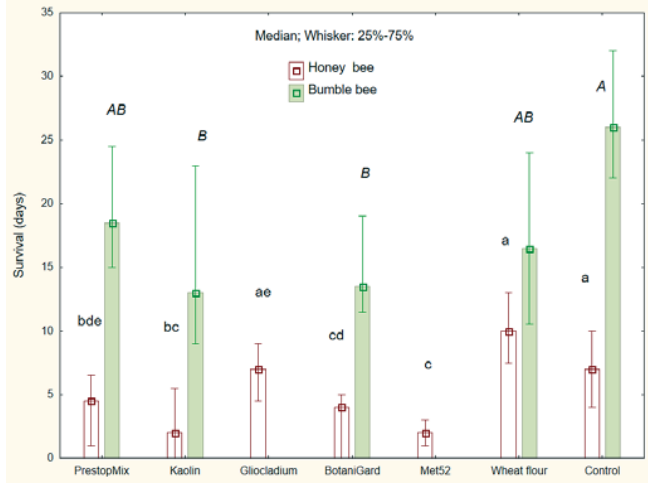

Figure 1 Mean survival of honey bees and bumble bees exposed to different biopesticides and inert materials. Letters indicate statistically significant (p<0.05) differences between treatments

Both metabolic rate and water loss rate in forced immobility are significantly lower in bumble bees compared to honey bees (MR: F(1;64)=3.9; p=0.05; WLR: F(1;64)=24.7; p<0.001). The MR of honey bees did not decrease in time (t=-0.37 df=3 p=0.74) as well did not change the WLR (t=0.68 df=3 p=0.55). In bumble bees, however the MR decreased significantly (t=7.18 df=5 p<0.001), whereas the WLR stayed unchanged (t=1.36 df=5 p=0.23) (see also Karise et al., 2016).

None of the biopreparations nor inert materials affected the metabolic rate of either of the species (F(4,42)=0.32, p=0.86), although the variation of the change rate was larger in honey bees compared to bumble bees (F(1,42)=7.39, p=0.009). There was no co-effect of species and treatment (F(4,42)=0.40, p=0.81).

Water loss rate, however, was significantly affected by treatment in both species (honey bee: F(6,29)=35.54; p<0.001; bumble bee: F(4,20)=6.75; p=0.001). We saw that kaolin and Prestop-Mix increased the water loss rate of either of bee species, BotaniGard increased it in honey bees, whereas powder of *G. catenulatum* spores, Met52 and wheat flour did not (Figure 2).

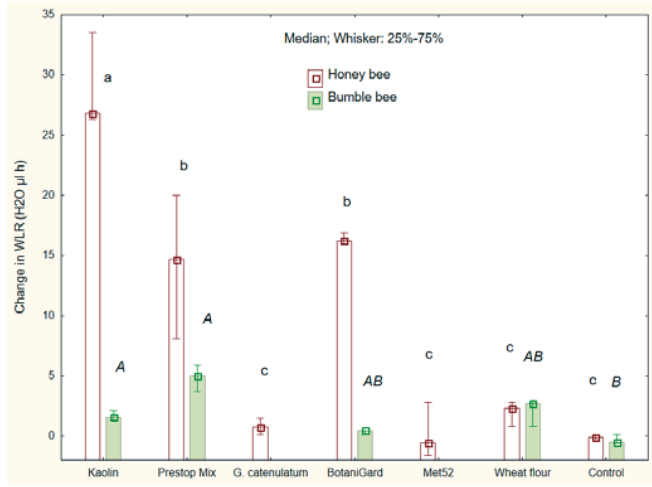

Figure 2 Mean change in water loss rate (WLR) after treatment with microbial biopesticides and inert powders. Letters indicate statistically significant ($p<0.05$) differences between treatments

Discussion

Measuring sub-lethal effects by means of respiratory physiology is effective and precise, however the technique has its limitations. The initial acquirement costs of the equipment would be high, however running the experiments would not cost much. Positive is that the technique allows to measure processes in a living intact organism and several characteristics in parallel, but demands individual measurements, which makes the process time-consuming.[6] In addition, the large variability of individuals makes detecting significant changes less achievable.

Honey bees and bumble bees are both social bee species, however their individual traits and species specific behaviour may differ largely. Bumble bees are considered as primitively eusocial, which differs by queen developmental pathway from advanced eusociality present in honey bees and ants.[12] We saw that bumble bees have lower metabolic rate than honey bees. This may be due to physiological properties or behavioural peculiarity. We saw, that bumble bees are able to calm down much faster. When forced to limited space, they stop struggling and eventually enter to deep resting state,[13,14] which is recognizable through presence of discontinuous gas exchange cycles in their respiratory patterns.[15,16] By honey bees we did not record discontinuous respiration cycles nor during 3h of pre-treatment period neither during the 3h course after the treatment. Treatment itself causes rapid increase of the activity level, which passes faster in bumble bees than in honey bees. We explain the difference in natural respiratory patterns and with the variable nature of bee species. Honey bee foragers are meant to fulfil the highly demanding foraging task for rapidly growing colonies, whereas for bumble bees this intrinsic pressure is lower. In addition, when it is too cold, honey bees use to cluster and heat themselves collectively,[17] when bumble bees are able to stay overnight alone out of hives.[18] Bumble bees´ ability to survive in unpleasant conditions is much better. This was seen also in our experiment. The measurements of MR in honey bees have shown, that in more favourable conditions they start respire discontinuously, too (unpublished observations of the authors). It is suggested that discontinuous respiration aids to diminish respiratory water loss.[15]

We saw variable effects of different microbial preparations on the studied bee species. Typically, honey bees´ reaction on treatments was stronger, however the trends were similar. Both entomopathogenic preparations affected honey bee and bumble bee survival. Biological fungicide Prestop-Mix, however affected significantly only honey bees and not bumble bees. The kaolin, an inert component of Prestop-Mix, affected significantly both bumble bee and honey bee

survival at the rate comparable with bioinsecticides. Kaolin and some other mineral powders are also used as insecticides against warehouse pest insects or to protect leaf and fruit surfaces from damages made by sucking insects.[19] We saw that the mineral powder may affect also bees, when they are delivering biological preparations to crops. Kaolin has been shown to change the lipid structure[20] on insect cuticle thus increasing the cuticular water permeability.[11] In our experiment the fine wheat flour did not affect the mortality, MR or WLR in either of bee species, which points out, that the mineral composition of kaolin rather affects insects than powder itself. The non-toxic microorganisms themselves do not affect the physiological processes of bees: no effect of pure *G. catenulatum* spores was detected on honey bee WLR, neither of Met52 which contains corn as carrier material. BotaniGard however contains mineral powder and affected honey bee WLR at the same rate than Prestop-Mix. The effect of treatments on bee WLR indicates that any preparation with corn as inert material is causing less stress to bees used in entomovectoring.

Conclusion

We saw that honey bees and bumble bees react similarly on microbiological preparations, however the reaction strength differed. Entomopathogenic preparations do affect the longevity of both bee species, in addition the inert powders also can do it. This should be taken into account when developing novel microbial preparations for entomovectoring systems. Comparison of these two bee species under stress from microbiological preparations revealed that bumble bees seem to suffer less. In addition, bumble bees suite better in analysing changes in respiratory patterns of bees.

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1.14 New working group – Testing side effects of microbials

Shannon Borges, Emily McVey, Jacoba Wassenberg

For the developments with this working group, see these proceedings:
Thomas Steeger - Working groups of the ICPPR Bee Protection Group – Developments and progress.

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2019 – 2021	RITA1/02-10-09 „Opportunities for mitigation of bee losses“
2015 – 2020	IUT36-2 „Sustainable crop protection: harnessing ecosystem services for plant production“
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2012 – 2015	ETF9450 “Impact of pesticide residues on the foraging behaviour and physiology of pollinators“

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2015-...	OÜ R-honey juhatuse liige
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Enesetäiendus ja koolitused:

2017	RNAi tehnoloogial põhinev jätkusuutlik kahjurite tõrje ja haiguste kontroll, Helsingi Ülikool

Teaduspreemiad ja stipendiumid:

SA Archimedes DoRa pluss lühiajalise õpirände 5 stipendiumi

- Osalemine konverentsidel 2016, 2017 (2x), 2018 (2x)

SA Archimedes Kristjan Jaagu välislähetuse stipendium
- Osalemine konverentsil 2017

ASTRA Väärtusahelapõhine biomajandus stipendium
- Osalemine konverentsil 2018

ASTRA väärtusahelapõhine biomajandus. Maateaduste ja ökoloogia doktorikool 2016-2022
- Osalemine kompleks ekspeditsioonil Reunioni saarele

Osalemine uurimisprojektides:

2019 – 2021	RITA1/02-10-09 „Mesilaste hukkumise vähendamise võimalused“
2015 – 2020	IUT36-2 „Jätkusuutlik taimekaitse: ökosüsteemi teenuste rakendamine taimekasvatuses“
2017 – 2018	P170058PKTK „Pestitsiidide ja patogeenide koostoime meemesilaste ja kimalaste füsioloogilistele näitajatele ja elueale“
2012 – 2015	ETF9450 “Pestitsiidijääkide mõju tolmeldajate korjekäitumisele ja füsioloogiale“

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3.1. A Chapter in a book

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Raimets, R; Karise, R; Mänd, M. 2017. Synergistic effects between variety of insecticides and an EBI fungicide combinations on bumble bees (*Bombus terrestris L.*).Estonian Beekeepers Association, 11.

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Raimets, R. 2016. Soome mesinikud väisasid Eesti Maaülikooli. Mesinik, 95, 10-10.

VIIS VIIMAST KAITSMIST

JIAYAN YE
QUANTITATIVE SCALING OF FOLIAGE PHOTOSYNTHETIC CHARACTERISTICS AND RELEASE OF CONSTITUTIVE AND INDUCED VOLATILES WITH SEVERITY OF BIOTIC STRESS
BIOOTILISE STRESSI KVANTITATIIVNE MÕJU LEHTEDE FOTOSÜNTEESILE NING KONSTITUTIIVSETE JA INDUTSEERITUD LENDUVATE ÜHENDITE EMISSIOONILE
Professor Ülo Niinemets
6. juuni 2019

KERSTI VENNIK
MEASUREMENTS AND SIMULATIONS OF RUT DEPTH DUE TO SINGLE AND MULTIPLE PASSES OF MILITARY VEHICLES ON TYPICAL ESTONIAN SOILS AND NATURAL RECOVERY OF RUTS
MILITAARSÕIDUKITE ÜLESÕITUDE TULEMUSENA KUJUNENUD ROOPASÜGAVUSE MÕÕTMINE JA MODELLEERIMINE EESTI MULDADEL NING ROOBASTE LOODUSLIK TAASTUMINE
Professor Endla Reintam, Thomas Keller, PhD (Rootsi Põllumajandusülikool), Peeter Kukk, PhD (Tartu Ülikool)
19. juuni 2019

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